The Role of PI3K Signaling in Neuroblastoma and Glioblastoma

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ABBREVIATIONS

4E-BP	4E-binding protein
AML	acute myeloid leukemia
AT/RT	atypical teratoid/rhabdoid tumors
ATP	adenosine triphosphate
cDNA	complementary DNA
CML	chronic myelogenous leukemia
CNS	central nervous system
DM	double minute
EGFR	epidermal growth factor receptor
Erk1/2	extracellular signal-regulated kinase 1/2
FKHR	forkhead in rhabdomyosarcoma
FRAP	FK506 binding protein 12-rapamycin associated protein 1
GB	glioblastoma
GPCR	G protein-coupled receptor
GSK-3β	glycogen synthase kinase-3β
HGF	hepatocyte growth factor
HSR	homogeneously staining region
IGF-I, IGF-II	insulin-like growth factor I, II
IGFBP	insulin-like growth factor binding protein
IGFR, IGF-IR	insulin-like growth factor receptor, insulin-like growth factor I receptor
INSS	International Neuroblastoma Staging System
IR	insulin receptor
IRS	insulin receptor substrate
LOH	loss of heterozygosity
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
mTORC2	mammalian target of rapamycin-rictor complex 2
NB	neuroblastoma
NCI	National Cancer Institute
PDGFR	platelet-derived growth factor receptor
PDK1	phosphoinositide-dependent kinase-1

PDK2	phosphoinositide-dependent kinase-2
PI	phosphatidylinositol
PI3K	phosphatidylinositol 3-kinase
PI(3)P	phosphatidylinositol 3-phosphate
PI(3,4)P_2	phosphatidylinositol (3,4)-bisphosphate
PI(3,4,5)P_3	phosphatidylinositol (3,4,5)-trisphosphate
PKB	protein kinase B
PTB	phosphotyrosine-binding
PTEN	phosphatase and tensin homolog
Rheb	Ras homologue enriched in the brain
RNAi	RNA interference
RT	radiotherapy
RTK	receptor tyrosine kinase
S6K	ribosomal protein S6 kinase
SCF	stem cell factor
SCLC	small cell lung cancer
SH2	Src homology-2
shRNA	short hairpin RNA
TSC1, TSC2	tuberous sclerosis complex 1, 2
VEGF	vascular endothelial growth factor
WHO	World Health Organization

1 A. Summary

A hallmark feature of human cancer is aberrant signaling from activated receptor tyrosine kinases (RTKs). Their localization in the cellular membrane allows them to translate environmental cues into intracellular signals, making them crucial components of signaling networks. Genetic alterations leading to over-expression or ligand-independent stimulation of RTKs are frequently observed in tumor cells and lead to deviant pathway activation. Targeted inhibition of tyrosine kinases with pharmacological inhibitors has had limited effects and few compounds have proven beneficial for large patient groups. Considering that tumors often express a number of different RTKs, an alternative approach would be to target downstream signaling pathways that are common to a variety of growth factor receptors. The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB, hereinafter Akt) pathway represents such a pathway. In view of the fact that this cascade is subject to numerous genetic alterations in human cancer, it provides a variety of potential therapeutic targets. The studies presented in this thesis were aimed at gaining a better understanding of the involvement of the PI3K family in different human cancers. This family of proteins consists of eight isoforms, which are grouped into three classes based on sequence homology and substrate specificity. While the catalytic class I_A member p110α has been the focus of much attention, the role of the other isoforms is less clear. Therefore, we aimed to evaluate the contribution of various PI3K family members to cellular responses in human cancer models.

The main aim of this study was to assess the role of individual PI3K isoforms in the biology of neuroblastoma (NB), a pediatric cancer that arises from neural crest cells. According to the NCI, NB accounts for 7-10% of all cancers diagnosed in children younger than 15 years of age. The enigmatic clinical behavior of this cancer ranges from spontaneous regression to rapid tumor progression, ultimately leading to death. Although a recent study reported a correlation between activation of the downstream signaling mediator Akt and poor prognosis, little is known about the involvement of this pathway in neuroblastoma. Therefore, we analyzed cell lines and patient samples for PI3K expression both at the mRNA and protein level. The class I_A isoform p110δ was found to be over-expressed both at the mRNA and protein level in a subset of primary NB tumors and cell lines. Interestingly, increased expression of p85α/p110δ was mainly detected in children under the age of one and was found to inversely correlate with *MYCN* amplification. Based on the observation that p110δ was highly over-expressed in primary tumors, cell lines were generated in which p110α or

p110δ were stably downregulated using RNAi. Cellular responses, such as basal proliferation and pathway activation upon growth factor stimulation were then compared between cells expressing decreased levels of p110α or p110δ and control cells. We could show that cells with high levels of p110δ, such as SH-SY5Y, greatly depend on this isoform for survival under limiting culture conditions and for the proliferative response to growth factors. These studies unveiled a novel role for p110δ in the regulation of NB proliferation and demonstrated that p110α and p110δ have non-redundant functions in these tumor cells.

Another project was aimed at investigating the effects of specifically inhibiting PI3K isoforms in glioblastoma (GB). These brain tumors have a highly invasive phenotype and are considered the most malignant type of glioma. Although a vast number of studies have provided evidence that the PI3K/Akt pathway plays a crucial role in the pathogenesis of brain tumors, the contribution of individual PI3K family members remains unclear. Our initial findings have highlighted unique contributions of individual isoforms to a variety of biological responses. Blocking the class II isoform PI3KC2β with a selective pharmacological inhibitor was found to inhibit the proliferation of GB cell lines and *ex vivo* cultures. Furthermore, co-treatment with the inhibitor sensitized cells to chemotherapeutic agents, such as doxorubicin. Preliminary results with inhibitors of the class I_A PI3Ks showed that inhibition of p110α attenuates activation of Akt and S6 kinase (S6K) in response to growth factor stimulation and affects anchorage-independent growth. The related isoform p110β also appears to contribute to anchorage-independent growth and cellular motility. The exact mechanisms underlying these differences are not yet understood.

Taken together, this dissertation explores the role of PI3K signaling in NB and GB. It highlights the functional differences of individual catalytic PI3K isoforms, thus confirming the idea that the members of this family of proteins can have specific and non-overlapping functions in cellular responses. The involvement of PI3K/Akt signaling in human malignancies has sparked interest in targeting PI3Ks as a novel therapeutic approach. However, the existence of eight isoforms necessitates a detailed understanding of the individual functions, thus delaying clinical application of isoform-specific PI3K inhibitors. Nevertheless, the wait could well pay off, considering that these proteins are expressed in a tissue-specific manner and seem to have non-redundant functions, thus making them very attractive molecular targets.

1 B. Zusammenfassung

Ein bezeichnendes Merkmal, welches bei Krebserkrankungen häufig beobachtet wird, ist die abnormale Aktivierung von Rezeptor-Tyrosinkinasen (RTK). Ihre Lokalisierung innerhalb der Zellmembran erlaubt es diesen Proteinen, Signale aus der Umgebung in intrazellulläre Antworten zu übersetzen, was sie folglich zu wichtigen Bestandteilen von Signal-Netzwerken macht. Genetische Veränderungen, welche zu Überexpression oder Ligand-unabhängiger Stimulierung von RTK führen, haben oftmals eine Überaktiverung von Signalkaskaden zur Folge. Gezielte Blockierung von Tyrosinkinasen mit pharmakologischen Inhibitoren als Krebstherapie hat bisher unbefriedigende Resultate ergeben, und nur wenige dieser Verbindungen haben sich für grosse Patientengruppen bewährt. In Anbetracht der Tatsache, dass Tumoren oftmals eine Vielzahl verschiedener RTK exprimieren, wäre ein alternativer Ansatz, Signalkaskaden, welche meheren RTK gemeinsam sind, ins Visier zu nehmen. Der Phosphatidylinositol 3-Kinase (PI3K)/Protein Kinase B (PKB, nachfolgend Akt) Signalweg stellt eine solche Kaskade dar. Da Proteine dieses Signalwegs oft von genetischen Veränderungen betroffen sind, bieten sich eine Vielzahl von interessanten Zielmolekülen an. Die vorliegende Arbeit sollte dazu beitragen, ein besseres Verständnis der Rolle der einzelnen PI3K in verschiedenen Krebsarten zu erlangen. Diese Proteinfamilie besteht aus acht Mitgliedern, welche je nach Vorkommen im Gewebe sowie Substratspezifität in drei Klassen unterteilt werden. Obschon der katalytischen Klasse I_A Isoform p110α viel Beachtung geschenkt wurde, bleibt die Rolle der anderen Mitglieder noch unklar. Unser Ziel war es daher, den Beitrag einzelner PI3K Isoformen an verschiedene zelluläre Vorgänge in menschlichen Krebszellen zu erörtern.

Das Hauptziel dieser Studie lag darin, die Rolle einzelner PI3K in der Biologie von Neuroblastoma (NB), einer pädiatrischen Krebsart, welche aus Zellen der Neuralleiste entsteht, zu ermitteln. Gemäss dem National Cancer Institute macht NB 7-10% aller Krebsdiagnosen in Kindern unter 15 Jahren aus. Das rätselhafte klinische Verhalten dieser Krebsart reicht von spontaner Regression zu schnellem Tumorwachstum, welches schlussendlich zum Tod führen kann. Obwohl eine kürzlich veröffentlichte Studie eine Korrelation zwischen Aktivierung von Akt, welches ein wichtiges Mitglied des PI3K Signalwegs darstellt, und schlechter Prognose aufzeigen konnte, bleibt die Rolle dieser Signalkaskade in NB noch unbekannt. Wir haben daher die Expression von PI3K in primären NB Tumoren und Zelllinien untersucht, sowohl auf der mRNA- wie auch auf der Proteinebene. Unsere Untersuchungen haben gezeigt, dass die Klasse I_A Isoform p110δ

sowohl auf der mRNA- wie auf der Proteinebene in einer Teilgruppe der Patienten überexprimiert ist. Interessanterweise wurde die Überexpression von p85α/p110δ vor allem bei Kindern, die zum Zeitpunkt der Diagnose noch nicht einjährig waren, nachgewiesen. Des weiteren konnten wir zeigen, dass zwischen der Expression von p110δ und *MYCN*-Amplifizerung eine negative Korrelation besteht. Aufgrund der Beobachtung, dass p110δ in primären Tumoren stark überexprimiert ist, wurden Zelllinien generiert, in welchen p110α oder p110δ stabil herunterreguliert war. Zelluläre Antworten wie Proliferation oder Aktivierung von Signalkaskaden nach Stimulierung mit Wachstumsfaktoren wurden daraufhin verglichen. In Zellen mit hoher p110δ Exprimierung, wie z.B. SH-SY5Y, konnten wir eine zentrale Rolle dieser Isoform in der Regulierung des Überlebens unter limitierenden Zellkultur Bedingungen, sowie für die Wachstumsfaktor-induzierte Proliferation nachweisen. Diese Studien haben eine neue Funktion von p110δ in der Regulierung der Proliferation von NB Zellen aufgezeigt. Des Weiteren konnten wir darlegen, dass p110α und p110δ nicht-überlappende Funktionen in diesen Tumorzellen haben.

In einem Nebenprojekt sollten die Auswirkungen der Inhibition einzelner PI3K Isoformen in GB untersucht werden. Diese Gehirntumoren zeichnen sich durch einen hoch-invasiven Phänotyp aus und stellen die bösartigste Form von Glioma dar. Obwohl eine Vielzahl von Studien aufgezeigt haben, dass der PI3K/Akt Signalweg eine wichtige Rolle in der Pathogenese von Gehirntumoren spielt, bleibt der Beitrag der verschiedenen PI3K Miglieder weitgehend unklar. In ersten Versuchen konnten wir zeigen, dass einzelne Isoformen in unterschiedlichem Ausmass an der Regulierung verschiedenster biologischer Vorgänge beteiligt sind. Die Hemmung der Klasse II Isoform PI3KC2β mit einem pharmakologischen Inhibitor führte zu einer verminderten Proliferationsrate von GB Zellen und *ex vivo* Kulturen. Des weiteren wurde die Sensitivität der Zellen auf Chemotherapeutika (z.B. Doxorubicin) bei gleichzeitiger Behandlung mit dem Inhibitor erhöht. Erste Experimente mit Inhibitoren der Klasse I_A PI3K zeigten, dass die Hemmung von p110α bei gleichzeitiger Stimulation mit Wachstumsfaktoren eine verminderte Aktivierung von Akt und S6 Protein zur Folge hat. Zudem wurde ein Einfluss auf Verankerungs-unabhängiges Zellwachstum beobachtet. Die verwandte Isoform p110β scheint ebenfalls an der Regulierung von Verankerungs-unabhängigem Zellwachstum und Zellmotiliät beteiligt zu sein. Die genauen Mechanismen, die zu den beobachteten Unterschieden führen, bleiben jedoch weitgehend unklar.

Zusammengefasst ermittelt die vorliegende Dissertation die Rolle des PI3K Signalwegs in NB und GB. Dabei werden die funktionellen Unterschiede der einzelnen katalytischen PI3K Isoformen aufgezeigt, was die weitläufig anerkannte Ansicht bestätigt, dass individuelle Mitglieder spezifische, nicht-überlappende Funktionen haben. Die Erkennung der zentralen Rolle des PI3K/Akt Signalwegs in der Krebsentstehung hat die PI3K als neue therapeutische Zielmoleküle hervorgehoben. Aufgrund der Existenz von acht Isoformen ist ein detailliertes Verständnis der individuellen Funktionen unerlässlich, was den Einsatz von Isoform-spezifischen PI3K in klinischen Studien jedoch verzöget. Nichtsdestotrotz deuten sowohl die gewebespezifische Exprimierung dieser Proteine, als auch die Tatsache, dass sie nicht-überlappende Funktionen haben, darauf hin, dass die PI3K geeignete Zielmoleküle sind und dass sich das Warten durchaus lohnen könnte.

2 Introduction

2.1 Neuroblastoma

2.1.1 Introduction

NB is a childhood neoplasm arising from neural crest cells. According to the NCI, NB accounts for 7-10% of all cancers diagnosed in children younger than 15 years of age. Two thirds of all cases present in children under the age of five. Tumors can arise anywhere along the sympathetic nervous system, with about half of all cases arising from the adrenal gland and the rest deriving from paraspinal sympathetic ganglia. The symptoms experienced at clinical presentation are variable and depend on the site of primary tumor origin. According to the NCI, approximately 70% of NB patients have metastatic disease at the time of diagnosis. A characteristic feature of this malignancy is the variability in clinical phenotype ranging from spontaneous regression to rapid tumor progression, ultimately leading to death.

Based on the observation that young children with localized tumors have a much better prognosis, various attempts have been made to screen infants in order to detect those with early onset disease. Although population-based screening trials were able to identify a considerable number of NB patients, the tumors detected by this method generally had favorable biological features and thus represented cases that presumably would have spontaneously regressed (1, 2). Therefore, there seems to be no general health benefit from screening newborns for neuroblastoma. Modern technologies, such as microarray analysis, have allowed genome-wide comparison of tumors of different stages in order to identify gene classifiers that could be useful for predicting clinical outcome and understanding the biology of tumor progression (3, 4). However, despite recent advances in understanding the biology of neuroblastoma, this childhood cancer remains enigmatic.

The etiology of NB is unclear (5). While most cases arise sporadically, a small fraction (1-1.5%) shows familial recurrence (6). To date, the gene (or genes) responsible for the development of disease have not been identified, due to a lack of familial clustering or the identification of informative families. Nevertheless, a number of characteristic genetic aberrations with prognostic value have been discovered during the past few decades (7). The most important alterations and their association with different patient groups are summarized in Table 2 (adapted from (5)) and discussed in more detail below.

In order to ensure accurate risk stratification and choice of therapy, precise staging of tumors is a prerequisite. In an effort to develop a classification scheme allowing uniform staging, the International Neuroblastoma Staging System (INSS) was developed (8, 9). The INSS largely depends on precise analysis of tumor location and lymph node involvement during surgery. This system has proven very reliable in staging of NB (10) and is currently used in nearly all standardized treatment protocols. An overview of stage characteristics is given in Table 1 (adapted from (9)).

Stage	Description
1	Localized tumor with complete gross excision, with or without microscopic residual disease. Ipsilateral lymph nodes negative for tumor microscopically
2A	Localized tumor with incomplete gross excision. Ipsilateral lymph nodes negative for tumor microscopically
2B	Localized tumor with or without complete gross excision. Ipsilateral lymph nodes positive for tumor, contralateral lymph nodes negative
3	Unresectable unilateral tumor infiltrating across the midline (defined as the vertebral column), with or without lymph node involvement. Or: Localized unilateral tumor with contralateral lymph node involvement. Or: Midline tumor with bilateral extension by infiltration or by lymph node involvement.
4	Any tumor with dissemination to distant lymph nodes, bone, bone marrow, liver skin and/or other organs
4S	Localized primary tumor in patients under one year of age with dissemination limited to skin, liver, and/or bone marrow

Table 1 INSS Staging System (adapted from (9)). This staging system relies on exact analysis of tumor location and lymph node involvement by the operating surgeon and has proven very reliable in NB staging prior to treatment.

A striking feature of NB is its ability to spontaneously regress, a phenomenon that was first described more than three decades ago (11). This situation is mainly observed in children with small localized tumors belonging to the 4S group, and can even include patients presenting with metastatic disease. Regression is usually observed in biologically and clinically favorable tumors which require minimal therapy (12). Although a number of hypotheses exist to explain this phenomenon (13, 14), the underlying mechanisms remain elusive.

2.1.2 Genetic Alterations

Frequently detected chromosomal aberrations in NB include both gains and losses of genetic material (15). A number of studies have demonstrated the prognostic value of cellular ploidy, particularly in infants under 1 year of age (16-19). To date, the cause(s) of this form of genetic instability as well as the implications for disease progression remain unclear. NB commonly display allelic loss of heterozygosity (LOH) within specific regions of chromosomes 1, 2, 3, 11, 12, 13, 14 and 15 (20, 21). Despite relentless efforts to uncover the link between these genetic alterations and disease progression, the exact underlying molecular mechanisms have not yet been deciphered. Nevertheless, LOH on chromosomes 1 and 11 has prognostic value, as it is observed more frequently in intermediate to high risk tumors (22). In addition, other chromosomal aberrations including translocations or amplification of genetic material have proven useful for risk stratification. The most well-known example is presumably amplification of *MYCN* which is detected in a considerable percentage of advanced stage tumors and is associated with an adverse outcome (23, 24).

	Low risk		**Intermediate risk**		**High risk**	
Clinical features						
Age	< 1 year	> 1 year	< 1 year	> 1 year	< 1 year	> 1 year
Stage	1, 2, 4S	1, 2	3, 4S	3,4	3, 4S	2, 3, 4
Genetic alterations						
Ploidy	triploid		diploid / tetraploid		diploid / tetraploid	
1p loss	rare		rare		frequent	
11q loss	rare		frequent		rare	
17q gain	rare		frequent		frequent	
MYCN	normal		normal		amplified	
Molecular alterations						
TrkA	high		low		low	
TrkB	low		low		high	
TrkC	high		low		low	
Prognosis						
5-year overall survival	95%		50%		25%	

Table 2 Risk stratification system for NB patients (adapted from (5)). This table gives an overview of some of the most common genetic alterations found in NB. Aside from age and stage, the existence of specific chromosomal abnormalities or expression of Trk-family members determine the risk group of a child and thus have an influence on the treatment regimen selected.

2.1.2.1 1p LOH

1p LOH is detected in nearly a third of all NB patients (22, 25). This alteration is associated with an unfavorable outcome, regardless of stage and has been shown to correlate with *MYCN* amplification (26). 1p36 deletions were first reported in NB in 1977 (27) and have since been the focus of much attention. The recurrent deletion of this region and its association with poor prognosis suggest that a putative tumor suppressor resides within this chromosomal area. Ideally, precise knowledge of the genes affected by this deletion could provide valuable information concerning the pathogenesis of neuroblastoma. Almost two decades ago, the main interest was in mapping the exact location of alteration, which has since been identified in the distal region of chromosome 1 and has been mapped to 1p36 (28-31). Around the same time, it was observed that cell lines and tumors harboring *MYCN* amplification show larger regions of deletion than those without this amplification (32, 33). More recent studies have been aimed at shedding some light on the genes located within the area of deletion. Modern screening methods, including microarray analysis, have allowed visualization of differentially expressed genes in patients harboring 1p LOH versus patients with an intact chromosome 1 (34-37). While some of the genes identified have known functions in neural differentiation, signal transduction in neural cells or regulation of the cell cycle (34-37), other genes have not yet been characterized. One such example is *CHD5*, which was identified in multiple analyses (38, 39). Using mouse models, this gene was recently shown to be a tumor suppressor that controls proliferation, apoptosis and senescence via the $p19^{Arf}$/p53 pathway (40). Of particular interest for the present study is the recent finding that the catalytic PI3K isoform p110δ is also among those genes found to be differentially expressed (35-37). This is not surprising, given that *PIK3CD*, the gene encoding this protein, lies at 1p36.2. This observation highlights the potential role this protein could play in the pathogenesis of neuroblastoma.

2.1.2.2 11q LOH

Nearly two decades ago, an increasing number of studies reported LOH for alleles on chromosome 11q (41, 42). The frequency of this genetic aberration is comparable to LOH at 1p and is found in nearly a third of the tumors analyzed (22, 41, 42). The prognostic value of 11q LOH has been studied extensively, in part because it inversely correlates with *MYCN* amplification (42, 43) and thus could provide a useful marker for aggressive tumors that lack *MYCN* amplification. Indeed, the prognosis for patients with tumors harboring an unbalanced deletion of 11q (11q loss with either retention or gain of 11p material) is very poor, and this alteration was shown to be an independent marker of decreased event-free and progression-free survival (22). As with 1p LOH, the finding that 11q LOH correlates with poor prognosis suggests the presence of a putative tumor suppressor gene within the area of deletion. Current investigations are aimed at identifying the genes affected by this loss of genetic information (44, 45).

2.1.2.3 17 gain

The most frequent genetic imbalances, seen in roughly half of all primary neuroblastoma, affect chromosome 17 (20, 21). The two types of alterations found are (i) whole chromosome 17 gain, which is seen more frequently in low grade tumors (20) or (ii) segmental gain of the long arm, which is observed in high grade tumors (20) and has been shown to predict an adverse clinical outcome (46, 47). Partial gain of chromosome 17q is mostly the result of an unbalanced translocation between 17q and a number of partners, including chromosomes 1p, 3p and 11q (48-52). Because this genetic rearrangement is detected so frequently, many attempts have been made to map the breakpoint, which has been found to be highly variable (53, 54). The exact mechanisms that lead to the adverse outcome in patients with 17q alterations remain yet to be unveiled. Recent attempts to identify genes that are overexpressed in tumors harboring 17q translocations have identified several potential candidates, including *BIRC5* (survivin) (55), *NME1* (56) and *PPM1D* (57). Although 17q gain is very strongly linked to both 1p deletion and *MYCN* amplification, it was shown to be a significant predictive factor in cases where neither of the latter alterations were detected (47). This finding is of particular interest, as it could facilitate the identification of more aggressive tumors in the intermediate risk group, thus leading to a more efficient treatment of certain patients.

2.1.2.4 *MYCN*

Early studies aimed at identifying genes associated with disease progression in NB reported the frequent amplification of chromosomal material, manifested as homogeneously staining regions (HSR) or double minute (DM) bodies (58-60). A proto-oncogene, termed *MYCN* due to its partial homology to *MYC* (encoding c-Myc), was found to be located within the amplified regions (61, 62). The *MYCN* gene is located on chromosome 2p and belongs to the family of Myc proteins, which function as transcription factors (63). Unlike c-Myc, which is ubiquitously expressed in proliferating tissues (64, 65), N-Myc expression seems to be limited to neuroectodermic and mesodermic cells, especially at early stages of differentiation (66). *MYCN* has been the focus of much attention, mainly because it was the first oncogene found to be of prognostic value for NB patients (23, 24, 67). Amplification of *MYCN* is detected in nearly a third of all primary NB (24) and is strongly associated with advanced stage disease and adverse prognosis (23, 67). This is reflected by the observation that over 40% of disseminated NB display *MYCN* amplification, whereas this rate is less than 10% for localized tumors (68). While the exact role of N-Myc in NB development remains unclear, it was shown that this protein regulates cell proliferation and differentiation (69-71), as well as invasiveness (72) and tumorigenesis (73). It was further shown that treatment of NB cells with 13-*cis*-retinoic acid, a vitamin A analogue known to induce neuronal differentiation of NB cells *in vitro* (74), leads to decreased levels of N-Myc (69, 75, 76). Considering the tremendous number of putative Myc family gene targets (77), it is not surprising that such a wide variety of cellular processes are affected by altered N-Myc expression levels in neuroblastoma.

2.1.3 Therapeutic Approaches

Prior to therapy, NB patients are assigned to different risk groups based on prognostic factors such as age at diagnosis, tumor biology and tumor stage (9, 78). While this risk stratification system has facilitated the selection of optimal treatment strategies, there is an ongoing interest to incorporate newly identified markers with prognostic value in order to further refine this classification system (79).

2.1.3.1 Low Risk Patients

Low risk patients include those younger than one year of age at the time of diagnosis with low grade tumors lacking *MYCN* amplification (9). According to the NCI, treatment of these patients usually involves surgical resection of the tumor with or without subsequent chemotherapy such as carboplatin, doxorubicin or etoposide. The outcome for this patient group is excellent as reflected by a disease-free survival rate of over 95% following surgery alone (80, 81). Treatment of patients with 4S tumors is largely dependent on clinical presentation. In some cases, children might only require supportive care, although low-dose chemotherapy or radiation can become necessary if complications, such as massive liver involvement, occur (12).

2.1.3.2 Intermediate Risk Patients

Intermediate risk patients are generally older than one year of age at the time of diagnosis and have intermediate or high grade tumors (9). While copy numbers of *MYCN* are typically normal, other genetic alterations, such as 11q loss or 17q gain are often observed (5). These patients usually undergo surgical resection of the tumor, followed by moderate chemotherapy (79). The survival rates in this risk group display considerable variation. While patients younger than one year of age display rates around 80%, this value ranges from 50% to 70% for older children (82, 83).

2.1.3.3 High Risk Patients

High risk patients are usually older than one year of age at the time of diagnosis and have intermediate or high grade tumors (5, 9). These tumors often harbor *MYCN* amplification as well as a number of other genetic alterations associated with unfavorable outcome (22-25, 47). Patients are typically treated with high-dose chemotherapy prior to surgical resection of the tumor (79, 84). In addition to myeloablative chemotherapy, whole-body irradiation and autologous stem cell transplantation, patients are frequently treated with 13-*cis*-retinoic acid in order to eliminate persistent minimal residual disease (79). Regardless of the extent of tumor

excision, patients also undergo radiation therapy. Despite aggressive treatment, the long-term survival rate for this patient group remains dismal (84-86).

2.2 Glioblastoma

2.2.1 Introduction

Brain tumors comprise a heterogeneous group of malignancies which pose a continuos challenge to clinicians due to their highly invasive nature and the restrictive properties of the blood-brain-barrier (87). According to the American Cancer Society, primary malignant tumors of the brain or spinal cord account for approximately 1.3% of all cancers in adults and are responsible for more than 2% of all cancer deaths in adults and children. Apart from primary tumors, a high incidence of secondary tumors can develop in the brain. These tumors originate from malignant tumors located elsewhere in the body and reach the brain via the bloodstream (88).

	Histology	**Percentage (%)**
Predominantly benign tumors	Meningioma	30.1
	Nerve sheath	8.0
	Pituitary	6.3
Gliomas	Glioblastoma	20.3
	Astrocytoma	9.8
	Oligodendroglioma	3.7
	Ependymoma	2.3
	Embryonal, including Medulloblastoma	1.7
Other tumors	Lymphoma	3.1
	Craniopharyngioma	0.7
	All other	13.9

Table 3 Distribution of all primary brain and CNS tumors by histology (adapted from (89)). Gliomas account for nearly 40% of all brain tumors. Together, astrocytomas and GBs make up the majority of all gliomas, with GBs accounting for over 50% alone.

2.2.2 Gliomas

Gliomas are tumors that originate from glial cells, i.e. astrocytes and oligodendrocytes (90). According to the Central Brain Tumor Registry of the United States, they account for 40% of all primary brain and central nervous system (CNS) tumors as illustrated in Table 3 (89). Together, astrocytomas and GBs make up the majority of all gliomas, with GBs accounting for over 50% alone (89). Other glial tumors include oligodendrogliomas, ependymomas and mixed gliomas, which consist of both astrocytic and oligodendroglial cells. Gliomas can arise in wide areas of the brain and more than half of all tumors originate in the frontal, temporal, parietal and occipital lobes (89).

Based on the World Health Organization (WHO) classification scheme, tumors are graded following analysis of nuclear atypia, mitoses, microvascular proliferation and necrosis in the most malignant region of the tumor (91). Prognosis for patients with low-grade gliomas is much better than for those with high-grade tumors, as illustrated by the 5-Year Survival Rates listed in Table 4 (89). However, despite relentless efforts to understand the molecular biology of GB, no curative therapy is available to date and treatment remains merely palliative (87).

WHO Grade	Histology Subtype	Percentage of Gliomas (%)	5-Year Survival Rate (%)
I	Pilocytic Astrocytoma	5.7	91.3
II	Oligodendroglioma	9.2	70.5
	Diffuse Astrocytoma	1.7	46.9
III	Anaplastic Astrocytoma	7.9	29.4
	Anaplastic Oligodendroglioma	5.1	40.1
IV	Glioblastoma	50.7	3.3
	Other Gliomas	19.7	

Table 4 Distribution of all primary brain and CNS gliomas by histology subtype (adapted from (89)). GBs account for roughly half of all gliomas and are by far the most aggressive subtype, as illustrated by the dismal 5-year survival rate of 3.3%.

2.2.2.1 Glioblastoma Multiforme

According to the WHO classification scheme, the most malignant type of glioma is grade IV GB multifome (hereinafter GB). This type of tumor is characterized by a highly invasive phenotype, which hinders complete surgical resection (92). Furthermore, grade IV tumors display rapid proliferation and are capable of forming new blood vessels in order to maintain their growth (93). Although GB can arise in patients of all age groups, the incidence is highest in the elderly (89). Primary GBs arise *de novo* from glial cells, most frequently in older patients with no previous history of low grade tumors. The genetic alterations associated with primary GBs include 10q LOH, over-expression or mutation of the epidermal growth factor receptor (EGFR), *p16* deletions and phosphatase and tensin homolog (*PTEN*) mutations (94). Secondary GBs are often seen in younger patients who were previously diagnosed with low-grade astrocytomas. These tumors typically display alterations of *TP53* and over-expression of the platelet-derived growth factor receptor α (PDGFRα) (94). As in most cancers, the pathogenesis of primary GB appears to be a multi-step process, involving a variety of genetic alterations (88). The distinct events underlying the development of primary or secondary GB ultimately lead to a disturbance of cell cycle control and aberrant RTK signaling. Therefore, it is not surprising that a vast number of studies have provided evidence that the PI3K/Akt pathway plays a crucial role in the pathogenesis of brain tumors (95-97).

2.2.3 Genetic Alterations

In view of the abundance of genetic alterations detected in GB, the following delineation is limited to alterations implicated in aberrant PI3K/Akt signaling.

2.2.3.1 10q LOH

Early analyses of chromosomal abnormalities in GB reported that 60% to 80% of all primary tumors harbored genetic alterations on chromosome 10 (98-100). The described events included both loss of an entire chromosome 10 or partial deletions (101). Although *PTEN* is a known target of deletion, the appearance of specific patterns of chromosomal loss suggests the presence of other tumor suppressor genes within chromosome 10 (102).

2.2.3.2 PTEN

Based on the high frequency of chromosomal irregularities involving chromosome 10, it was assumed that a putative tumor suppressor gene resided within this area. In 1997, two independent groups identified a gene within the region 10q23 (103, 104) which has since been shown to encode PTEN, a dual-specificity phosphatase capable of dephosphorylating both 3-phosphoinositides and tyrosine-phosphorylated proteins (105). This protein mainly acts as a PI3K antagonist and is involved in the regulation of many essential cellular functions, such as cell cycle progression, cell migration and apoptosis (105). Studies aimed at identifying *PTEN* mutations in GB have reported that 50% to 70% of primary GBs have *PTEN* deletion, mutation or loss of PTEN expression, implicating *PTEN* as one of the most affected genes in this disease (103, 106, 107). Various studies have demonstrated a correlation between loss of *PTEN* and Akt activation (95, 97), confirming that the PI3K/Akt pathway is deregulated in the absence of functional PTEN.

2.2.3.3 EGFR

More than two decades ago, a large number of studies reported genetic alterations and varying expression levels of genes encoding RTKs in glioma cell lines and primary tumors (108-112). To date, one of the most intensively studied RTKs is the epidermal growth factor receptor (EGFR). Signaling from this RTK has been shown to be disturbed by a number of mechanisms. *EGFR* gene amplification is observed in 30% to 50% of GBs and leads to protein over-expression in the majority of cases (108-110). Deletion mutations are also a frequent occurrence in GB. The most prominent deletion leads to the generation of a truncated receptor (termed EGFRvIII) which displays ligand-independent activation (113, 114). Immunohistochemical analysis of a microarray comprising 45 GB cases demonstrated that expression of the EGFRvIII mutant is correlated with elevated Akt activation, and therefore represents an alternate mechanism of Akt activation in the presence of wild-type PTEN (95). Furthermore, aberrant signaling via the EGFR can be attributed to the establishment of autocrine growth factor loops, which have been detected in glioma cells (115, 116). In summary, a number of mechanisms can lead to deviant pathway activation emerging from the EGFR.

2.2.3.4 PDGFR

PDGF plays a crucial role in normal developmental processes of the central nervous system (111). Therefore, it is not surprising that deregulated signaling involving this growth factor system has been linked to GB progression. Around the same time interest in the EGFR was sparked, a number of studies reported the expression of both the PDGFR and its ligand (112, 117), suggesting the presence of an autocrine or paracrine signaling loop. With the help of specific PDGFR inhibitors, recent studies have confirmed the existence of self-sufficient PDGF signaling in GB (118). Although the exact mechanisms remain elusive, it seems that amplifications (119) and activating deletions (120) can contribute to aberrant PDGF signaling.

2.2.3.5 IGF

Insulin-like growth factor-I and –II (IGF-I and IGF-II) are growth factors that are known to stimulate proliferation in several mammalian cell systems (121). IGF-signaling involves a complex network of growth factors and receptors as well as IGF binding proteins (IGFBP), which can sequester IGFs, thus affecting their half-lives and bioavailability (122). Unlike a number of other growth factors, IGFs circulate in the bloodstream and can therefore act as endocrine factors (122). Expression of the IGF receptors (IGFR) in human brain tissue was identified more than two decades ago (123, 124). This family of growth factors plays an important role in the growth and differentiation of the CNS (125, 126) and it is therefore not surprising that deregulated IGF signaling has been found to contribute substantially to the development of brain tumors (127, 128). Two recent studies have demonstrated that different mechanisms of aberrant IGF signaling can lead to activation of the PI3K/Akt pathway in GB. In one report it was shown that expression of IGFBP2 plays a key role in activating Akt (129). Another study provided evidence that the IGF-II/PI3K signaling axis is involved in autocrine signaling, thus promoting the growth of a subclass of highly aggressive gliomas lacking *EGFR* amplification (130). In summary, deregulated IGF signaling plays a crucial role in gliomagenesis and has recently been linked to aberrant PI3K/Akt pathway activation (128-130).

2.2.4 Therapeutic Approaches

To date, no curative therapy is available for GB (87). Nevertheless, a broad spectrum of techniques is available to prolong the survival time of patients. Aside from classical approaches such as surgical resection (131) and radiotherapy (132), a number of novel, more targeted therapeutic strategies have proven beneficial (reviewed in (87)). Frequently, different approaches are combined in order to improve therapeutic outcome (reviewed in (133)). Based on tumor grade and symptoms experienced by the patient, different treatment strategies are available. While surgical removal is recommended for most gliomas, radiotherapy and chemotherapy options depend on tumor stage and location (87).

2.2.4.1 Surgical Resection

Due to the highly invasive nature of GB cells, complete surgical resection of tumors is nearly impossible (92) and adjuvant therapies are indispensable. Nevertheless, surgery remains very important in GB therapy (131). Aside from its crucial role for final diagnosis, tumor excision helps alleviate symptoms caused by pressure on parts of the brain. Furthermore, it has been shown that extensive resection is a good prognostic indicator for GB patients (131).

2.2.4.2 Radiotherapy

Postoperative radiotherapy (RT) has become an integral part of standard treatment based on the observation that it has a beneficial effect on patient survival (132, 134, 135). A variety of radiation strategies are available, ranging from whole brain RT to partial RT and including different fractionation schemes. A study performed more than two decades ago describing the appearance of recurrent tumors within or in close proximity to the original site of tumor origin (136) substantiates the preference of partial RT over whole brain RT. Partial RT has the advantage of limiting the area of exposure to radiation, thus reducing the occurrence of side effects.

2.2.4.3 Chemotherapy

While surgical resection and postoperative RT form integral parts of current GB treatment, the role of chemotherapy is controversial. Although the effects of adjuvant chemotherapy are very modest and the ability of some agents to cross the blood-brain barrier is questionable, meta-analysis of 12 randomized clinical trials confirmed a beneficial effect of chemotherapy on patient survival (137). Novel antineoplastic agents, such as Temozolomide, have shown remarkable activity against gliomas, are able to cross the blood-brain barrier and have limited side effects (138). While the overall effect of chemotherapy is modest in patients with high-grade gliomas, a subgroup (mainly including patients with stage III anaplastic oligodendrogliomas) shows a significant response to treatment. Comparison of responders and non-responders has allowed the identification of molecular predictors of chemosensitivity (139). Their feasibility is currently under investigation. The observation that a subgroup of patients can indeed benefit from chemotherapy and the availability of novel agents displaying acceptable tolerability have increased the importance of chemotherapy in GB management (140, 141).

2.3 Phosphatidylinositol 3-Kinases

2.3.1 The Family of PI3Ks

The PI3Ks are a family of evolutionary conserved lipid kinases, which play a crucial role in controlling a wide variety of intracellular signaling events. PI3Ks phosphorylate phosphatidylinositol (PI) on the D-3 position of the inositol ring, producing distinct second messengers such as PI(3)P (PI(3)P), PI(3,4)P_2 (PI(3,4)P_2) and PI(3,4,5)P_3 (PI(3,4,5)P_3) (142). These second messengers are known to activate a number of proteins involved in complex signaling cascades, ultimately resulting in the regulation of cellular responses including growth, proliferation, survival and motility (141). A variety of specialized domains have been identified that are involved in recruiting these downstream signaling proteins to the membrane (143). The family of PI3Ks consists of eight isoforms, which are grouped into three classes, based on sequence homology and *in vitro* substrate specificity (144). To date, it is unclear if each isoform targets a specific downstream effector or if a certain degree of redundancy exists. An overview of the various isoforms as well as their substrate specificity and tissue distribution is given in Figure 1.

2.3.1.1 Class I

Class I_A includes the three catalytic subunits p110α, p110β, and p110δ (114). This class is known to form heterodimeric complexes with a p85 (p85α or p85β), p55 (p55α or p55γ) or p50 (p50α) regulatory subunit containing Src-homology 2 (SH2) domains. Sequence comparison of the catalytic subunits revealed high homology among the proteins. Class I_A PI3Ks are important mediators of signal transduction from activated RTK. The regulatory subunits are recruited to activated RTKs by means of two SH2 domains, which recognize specific phosphotyrosines within the cytoplasmic domain of the receptor (142). While the expression of p110α and p110β is ubiquitous, p110δ expression seems to be more tissue specific (142, 145). High levels of this isoform have mainly been reported in cells of the hematopoeitic system. However, p110δ has also been detected in cells of breast or melanocytic origin (145-148).

Class I_B is made up of p110γ and a regulatory subunit p101 (149) or p84 (150). Activation of p110γ is controlled by receptors capable of activating heterotrimeric guanine nucleotide-binding proteins, termed G protein-coupled receptors (GPCRs) (149). The PI3K

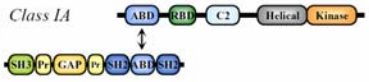
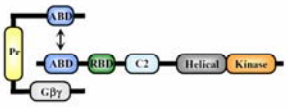
p110γ is thought to link GPCR signaling to PI(3,4,5)P_3 production, governing cell motility in inflammatory cells such as macrophages and to some extent in neutrophils (151).

2.3.1.2 Class II

Class II of PI3K comprises the three isoforms PI3KC2α, PI3KC2β and PI3KC2γ which differ from class I enzymes in size and substrate specificity *in vitro* (142, 144). The hallmarks of class II family members are a substrate specificity restricted to PI and PI(4)P *in vitro*, and a C-terminal C2 domain. Although the precise cellular function of these enzymes remains generally unknown, recent reports have described class II PI3Ks as downstream transducers of activated polypeptide growth factor receptors (152, 153) and as regulators of cell migration (154, 155).

2.3.1.3 Class III

Class III includes the mammalian homolog of the yeast vesicular protein-sorting protein Vsp34 (156). The major function of this isoform seems to be in intracellular trafficking events (157). More recently, Vsp34 has been found to be an activator of autophagy, contributing to early steps of autophagosome formation (158).

Class I	**Subunits** Regulatory	Catalytic	**Substrate Specifity**	**Activator**	**Tissue Distribution**
Class IA 	p85α p85β p55α p55γ p50α	p110α p110β p110δ	PI PIP PIP_2	Receptor tyrosine kinases Ras	p110α, p110β: ubiquitous p110δ: leukocytes
Class IB 	p101 p84	p110γ	PI PIP PIP_2	G-protein-coupled receptors (Gβγ) Ras	leukocytes
Class II					
		PI3KC2α,β,γ	PI PIP	Receptor tyrosine kinases G-protein-coupled receptors	PI3KC2α,C2β: ubiquitous PI3KC2γ: liver
Class III					
	p150	Vps34p analogues	PI	Constitutively active G-protein-coupled receptors (Gα)	

ABD – Adaptor Binding Domain
RBD – Ras Binding Domain
C2 – C2 Domain
Helical – Helical Domain
Kinase – Kinase Domain
SH3 – Src Homology Type 3 Domain
Pr – Proline-rich Domain
GAP – Bcr/Rac GAP Homology Domain
SH2 – Src Homology Type 2 Domain
Gβγ – Gβγ-binding Site

Figure 1 Overview of PI3K isoforms. The PI3K family consists of eight isoforms, which are grouped into three classes, based on sequence homology and *in vitro* substrate specificity.

2.4 Signaling from Activated Receptor Tyrosine Kinases

2.4.1 Activation of Receptor Tyrosine Kinases

RTKs play an essential role in cellular signaling and are involved in the regulation of many normal physiological processes, such as cell proliferation (159) and survival (159) as well as cell cycle control, cell adhesion and migration (160). Numerous RTKs have been identified and are classified based on ligand preference, induction of biological responses and primary structure (161). Their localization in the cellular membrane allows them to translate environmental cues, such as peptides, proteins, lipids, or carbohydrates into intracellular signals thus determining the biological outcome (161).

RTKs belong to the family of protein tyrosine kinases, which posses an intrinsic protein kinase activity, allowing the transfer of phosphate from adenosine triphosphate (ATP) to tyrosine residues of protein substrates (162). Tight control of receptor activity is guaranteed by protein-tyrosine phosphatases, by serine/threonine kinases as well as autoregulatory mechanisms within the receptors (161, 162). Upon ligand binding, conformational alterations are induced, allowing receptor dimerization and, ultimately, autophosphorylation of specific tyrosine residues within the cytoplasmic domains (163). Tyrosine autophosphorylation stimulates the intrinsic kinase activity of RTKs and leads to the generation of specific recruitment sites for important signal transducers (163). Downstream signaling is initiated when molecules bearing specialized phosphotyrosine-recognition domains, such as SH2 (164) or phosphotyrosine-binding (PTB) (165) domains bind to phosphotyrosine residues within the receptor. These signaling molecules are either adaptor proteins that contain additional protein-protein interaction motifs (e.g. Grb2 or Shc), proteins whose activity is stimulated upon association with the receptor (e.g. PI3Ks or PLCγ) or docking proteins, which bear domains allowing interaction with the activated receptor, the cellular membrane as well as other proteins (e.g insulin receptor substrate (IRS)). The unique combination of proteins recruited to the receptor thus determines the specific signaling cascade activated upon ligand binding.

2.4.2 PI3K Signaling from Activated Receptor Tyrosine Kinases

As mentioned above, RTKs undergo autophosphorylation of tyrosine residues within the intracellular domain following ligand binding, thus generating docking sites for proteins with specific recognition domains. Class I_A PI3Ks are important mediators of downstream signaling and can be activated by different mechanisms, all involving the association of proteins bearing specialized domains with phosphorylated tyrosine residues within RTKs (166). Direct interaction with these residues is mediated by SH2 domains of the p85 regulatory subunit (167), while indirect interaction involves scaffolding proteins and the adaptor protein Grb2 or IRS, which can associate with p85 (168). Binding of the regulatory subunit to the receptor triggers activation of the catalytic PI3K subunit p110, leading to a sharp increase in cellular 3-phosphoinositides. The second messenger $PI(3,4,5)P_3$ is the main effector of PI3K signaling and serves as a docking site for proteins bearing a pleckstrin homology (PH) domain (169). Two examples of proteins containing a PH domain are 3'-phosphoinositide-dependent kinase-1 (PDK1) or Akt. Upon binding to $PI(3,4)P_2$ or $PI(3,4,5)P_3$, Akt undergoes a conformational change, exposing two amino acids crucial for protein activation (170). Thr308 is located within the kinase domain and is phosphorylated by PDK1, thus stabilizing the activation loop (171). Since the kinase activity of PDK1 is not influenced by its binding to $PI(3,4,5)P_3$, the PH domain seems to mainly ensure the proximity of this kinase to its substrate (172). The other amino acid, Ser473, lies within the C-terminal domain and requires phosphorylation by a different kinase for full activation of Akt (170). A variety of kinases have been suggested to be involved in activating Ser473, such as PDK1 (173), integrin-linked kinase (174, 175), Akt itself (176) or, more recently, the mammalian target of rapamycin (mTOR)-rictor complex 2 (mTORC2) (177). This kinase is most frequently referred to as phosphoinositide-dependent kinase-2 (PDK2) (178). Although a wide variety of intracellular proteins contain lipid-binding domains, Akt is thought to be the main mediator of PI3K signaling due to its crucial role in regulating downstream substrates thus controlling essential cellular functions such as apoptosis, cell cycle progression, transcription and protein translation (141). Although the complete spectrum of proteins involved in PI3K/Akt signaling may not be completely understood, a number of important mediators are known, including tuberous sclerosis complex 1 and 2 (TSC1, TSC2), Ras homologue enriched in the brain (Rheb), mTOR, 4E-binding protein (4E-BP), ribosomal protein S6 kinase (S6K), BAD and glycogen synthase kinase-3 (GSK-3) (142). An overview of the pathway is given in Figure 2.

While activating signals are crucial to most biological processes, the abrogation of these signals is just as important to ensure proper cellular control. The main antagonist of PI3K signaling is the phosphatidylinositol 3-phosphatase PTEN. This phosphatase can specifically hydrolyze the D3 phosphate, thus controlling the levels of second messengers generated. (179, 180). The importance of this protein in ensuring accurate cellular functioning is highlighted by the high frequency of *PTEN* mutations in a number of human cancers, as discussed in 2.5.2.1 (103, 104).

Although PI3K signaling plays an important role in transmitting signals from activated RTKs, it is not the only pathway that is activated upon ligand binding. Another key pathway involves the mitogen-activated protein kinase (MAPK) cascade (181). All eukaryotic cells possess multiple MAPK pathways, which are divided into different groups based on sequence similarity (182). Although MAPKs can be activated by a wide variety of stimuli ranging from growth factors to cellular stress (183), each family is composed of a set of three evolutionarily conserved, sequentially acting kinases: a MAPKK kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK (182). The cascade comprised of Raf, MEK and extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2) plays an essential role in relaying signals from activated RTKs. Upon ligand binding, this pathway is activated by the GTPase Ras, which can bind directly to Raf (184). Activated ERK1 and ERK2 have been shown to phosphorylate a variety of substrates including membrane proteins, nuclear substrates, and cytoskeletal proteins (185). An overview of the pathways involved in transmitting signals from activated RTKs is given in Figure 2.

As a greater understanding of signaling from activated RTKs arises, it has become increasingly clear that these cascades do not form linear pathways, but instead constitute complex signaling networks, converging to activate downstream substrates (186) or regulating each other’s activity (187).

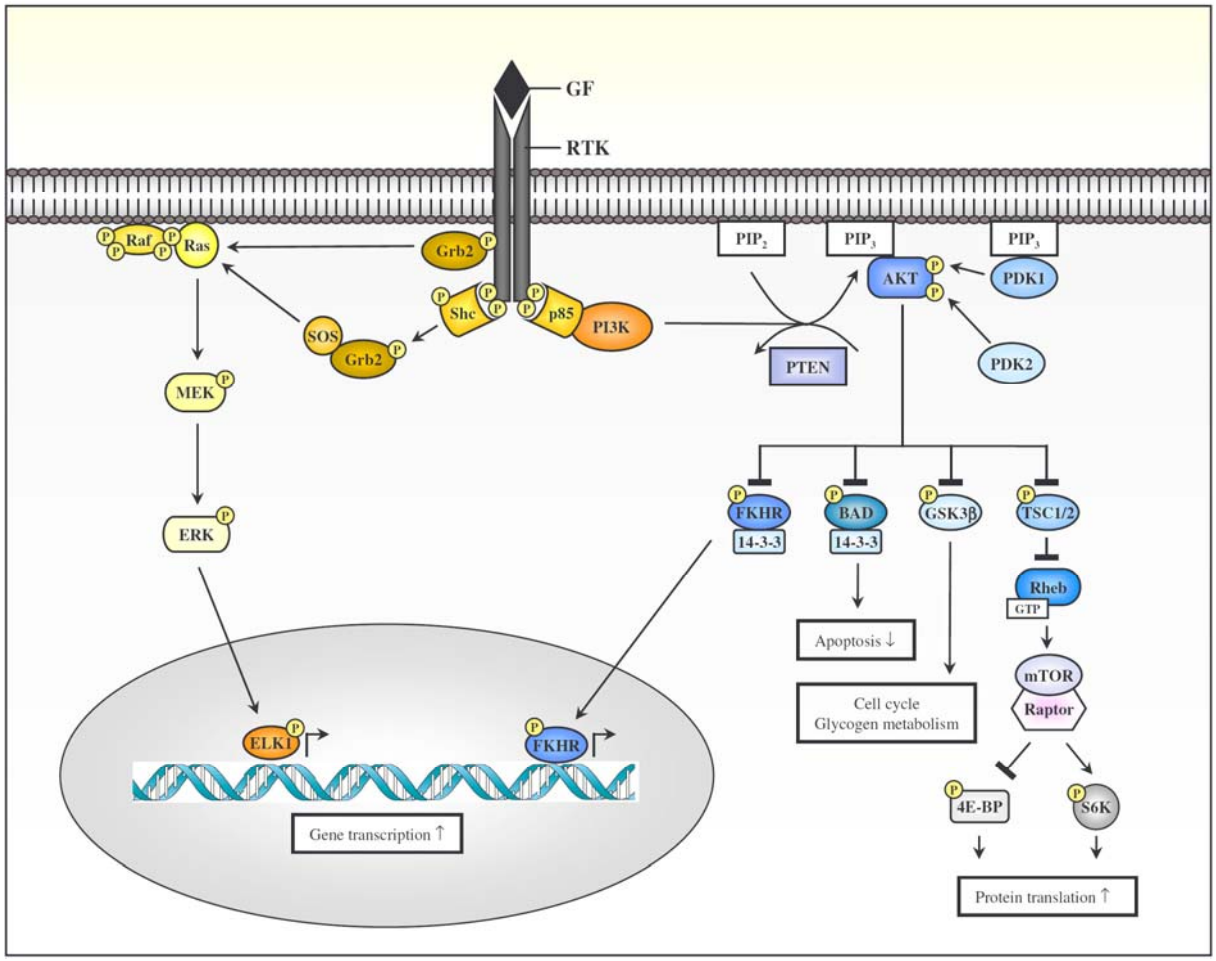


Figure 2 Schematic overview of signaling from activated RTKs. Upon ligand binding, the transmembrane receptor is activated by autophosphorylation, creating binding sites for adaptor proteins, such as the Src-homology collagen protein (Shc) or growth factor receptor-bound protein 2 (Grb2). Signals are transduced via the MAPK or the PI3K pathway. Key upstream regulators of MAPK signaling include Grb2, Son of Sevenless (SOS), Ras, Raf, MAPK/Erk kinase (MEK), extracellular signal-regulated kinase (ERK). Activation of the PI3K pathway is initiated by recruitment of the PI3K complex to the phosphorylated receptor through interactions via the regulatory subunit p85. Downstream signals are transduced via phosphoinositide-dependent kinase-1 and -2 (PDK1 and PDK2), protein kinase B/Akt (AKT), forkhead (FKHR), BAD, glycogen synthase kinase-3β (GSK3β), tuberous sclerosis complex (TSC1/2), Ras homologue enriched in the brain (Rheb), the mTOR-Raptor complex, 4E-binding protein (4E-BP) and ribosomal protein S6 kinase (S6K).

2.5 Genetic Alterations of PI3K Isoforms and Their Implication in Cancer

2.5.1 Introduction

Considering the crucial role PI3K/Akt signaling plays in regulating cell survival, it is not surprising that genetic alterations leading to increased pathway activation are detected in various human malignancies (188-190). In fact, the PI3K/Akt cascade is now known to be one of the signaling systems most frequently targeted by mutations or amplifications in human cancers (188). This finding has put PI3K/Akt signaling in the spotlight and has led to the emergence of a plethora of studies analyzing the mechanisms leading to deregulation of this pathway in malignant cells. Unfortunately, the majority of publications focus only on *PIK3CA*, completely disregarding the other PI3K isoforms. An overview of genetic alterations pertaining to components of the PI3K pathway in NB and GB is given in Table 5.

2.5.1.1 *PIK3CA*

Mutation of *PIK3CA*

A landmark study by Samuels et al. performed on a variety of different tumor types found that a high percentage of colorectal cancers, GBs, breast and gastric cancers harbored mutations in the *PIK3CA* gene (191). Genetic alterations in this gene were also detected in lung cancer, albeit at much lower rates (191). A large number of studies have since reported the existence of *PIK3CA* mutations in a variety of human malignancies (192-194). Interestingly, around 80% of the mutations map to one of three known hot spots, which are characterized by substitution of a single amino acid: E542K, E545K and H1047R. Studies aimed at characterizing these mutants have demonstrated their oncogenic potential (195-197).

Neuroblastoma To date, a single study has investigated the rate of *PIK3CA* mutations in NB. Two mutations were found in a total of 69 primary tumor samples, indicating that this genetic alteration is rare in NB (198). Although the alterations detected did not lie within the known hot spots, they were found to be located within the kinase or helical domains, implicating a possible increase in p110α activity.

Glioblastoma In GB, the values pertaining to *PIK3CA* mutations range from 0% (n=70) (199) to 27% (n=15) (191). The most consistent values seem to be around 5%, a number reported by various groups (200-203). A slightly higher rate of 18% was detected in a panel of primary tumors comprising 38 samples (204). The large discrepancies observed among these studies can possibly be explained by (i) the samples sizes, which are usually fairly small, (ii) by the composition of the samples, which in some cases contain both adult and pediatric tumor samples or (iii) the methods of detection, which can display different sensitivities. Barring the inconsistent rates of mutation, a common finding is that mutations in *PIK3CA* and *PTEN* seem to be largely mutually exclusive in GB (200-203).

Amplification of *PIK3CA*

Comparatively few studies have been aimed at evaluating the incidence of *PIK3CA* amplifications in human cancer. Nevertheless, amplification seems to play a role in the pathogenesis of certain malignancies, such as thyroid, lung or ovarian cancer (205-207) and could provide an alternative mechanism of pathway activation in the absence of mutations. This idea is substantiated by the observation that amplification of *PIK3CA* is a rare event in breast cancer, where mutation of this gene is one of the most frequent genetic alterations detected (208, 209).

Neuroblastoma Despite the growing interest in linking *PIK3CA* alterations to the pathogenesis of various human cancers, to date, not a single study has assessed the amplification rates of this gene in NB. A single research paper evaluating genomic DNA alterations in a panel of 42 NB cell lines reported no significant amplification of the *PIK3CA* locus (3q26) (210). Therefore, the contribution of p110α to the development of this pediatric cancer remains yet to be investigated.

Glioblastoma In GB, numbers concerning *PIK3CA* amplification rates are as diverse as those regarding mutation rates. While most groups have reported the total absence of *PIK3CA* amplification (200, 202, 204, 211), a few studies have demonstrated increased copy numbers of this gene (203, 212). However, the relevance of this genetic alteration for gliomagenesis remains to be evaluated.

2.5.1.2 *PIK3CB*

Very little is known about genetic alterations of *PIK3CB* in human cancer. However, despite its chromosomal proximity to *PIK3CA*, this isoform does not seem to be the target of mutational events (191), as illustrated by analysis of a large number of primary tissues and cell lines derived from malignant tumors (www.sanger.ac.uk/perl/genetics/CGP/cosmic).

Neuroblastoma There is currently no data available concerning *PIK3CB* mutation or amplification rates in NB.

Glioblastoma The only study aimed at investigating *PIK3CB* mutations in GB reported no mutation of this isoform upon sequence analysis of 10 primary tumors (212).

2.5.1.3 *PIK3CD*

Although *PIK3CD* does not seem to be frequently targeted by mutations, as exemplified by large-scale mutational analysis (www.sanger.ac.uk/perl/genetics/CGP/cosmic), alterations in gene copy numbers and mRNA levels could be involved in the development of certain cancers, including NB and GB.

Neuroblastoma Recent microarray-based studies performed on primary NB samples have reported decreased expression of *PIK3CD* in distinct patient groups, such as those harboring 1p LOH (36) or those with unfavorable biology (37). Interestingly, in tumors with amplification of *MYCN*, an important prognostic marker, levels of *PIK3CD* mRNA were found to be decreased (37). Analysis of primary NB samples included in the present thesis revealed that elevated mRNA levels of *PIK3CD* correlate with patient age (<1 year of age). Furthermore, mRNA levels were shown to be predictive of protein levels. A single study has investigated the mutational status of *PIK3CD* in NB. This report found that 2/46 primary NB samples harbored genetic alterations in the gene encoding p110δ (213). However, the biological consequence of these mutations remains to be investigated.

Glioblastoma A study comprising 103 GB tumor samples found that the mRNA levels of *PIK3CD* were elevated, despite normal copy numbers (211). A much smaller study encompassing 10 primary tumor samples subsequently found balanced copy number increases in *PIK3CD* (212).

2.5.1.4 *PIK3R1/PIK3R2*

Reports on the involvement of the regulatory PI3K subunits in cancer progression are quite rare. Nevertheless, it has been shown that some primary human colon and ovarian cancers harbor mutations in *PIK3R1*, producing deletions within the inter-SH2 region, thus leading to PI3K activation (212, 214).

Neuroblastoma To date, no data is available pursuant to *PIK3R1* or *PIK3R2* mutation or amplification rates in NB. However, expression profiling of 68 neuroblastoma samples revealed decreased expression of *PIK3R1* in stage 4 patients (4).

Glioblastoma Analysis of copy numbers and expression levels of *PIK3R1* and *PIK3R2* in GB revealed no differences when compared to non-neoplastic human brain tissue (211). A study encompassing 30 GB samples detected a deletion mutation in one sample (212). This mutation is predicted to give rise to a C-terminal deletion, possibly conferring oncogenic potential to the truncated protein (212).

2.5.1.5 *PIK3C2A/PIK3C2B*

Despite their established role in transducing signals from activated RTKs, very little is known about genetic alterations of the class II isoforms PIK3C2α and PIK3C2β in cancer (215). Large-scale mutational analysis revealed that mutations in *PIK3C2A* and *PIK3C2B* are nearly absent in most cancers (www.sanger.ac.uk/perl/genetics/CGP/cosmic) (191).

Neuroblastoma There is currently no data is available regarding genetic alterations of *PIK3C2A* or *PIK3C2B* in NB.

Glioblastoma In GB, 6% of tumor samples were found to have amplification of *PI3KC2B*, accompanied by an almost identical rate of mRNA over-expression (211). To date, no mutational analysis of class II PI3Ks has been performed in GB.

2.5.2 Alterations of Genes Involved in PI3K Signaling

2.5.2.1 *PTEN*

As discussed in chapter 2.2.3.2, *PTEN* is a firmly established tumor suppressor gene frequently involved in deregulated PI3K signaling. Genetic alterations involving this gene are implicated in the progression of a variety of human malignancies (105).

Neuroblastoma Although genetic alterations of *PTEN* play a key role in the progression of various human cancers, detailed analysis of a panel of NB cell lines led to the conclusion that disruption of the *PTEN* gene is not a frequent event in this cancer (216).

Glioblastoma The observation that 50% to 70% of all primary GBs have *PTEN* deletion, mutation or loss of *PTEN* expression, implicates *PTEN* as one of the most affected genes in this disease (103, 106, 107). While genetic alterations of *PTEN* are prevalent in adult GB, they are nearly absent in pediatric brain tumors (217), suggesting that the underlying mechanisms of tumor development are different in pediatric and adult cancer.

		Neuroblastoma	**Ref.**	**Glioblastoma**	**Ref.**
PIK3CA	*mutation*	low rates	(198)	varying rates (0%-27%)	(191, 199-204)
	amplification	no amplifications	(210)	varying rates (0%-50%)	(200, 202-204, 211, 212)
PIK3CB		no information		no mutations	(212)
PIK3CD		decreased mRNA levels rare mutations	(36, 37, 213)	increased mRNA levels, amplification	(211)
PIK3R1 *PIK3R2*		no information		mutation of *PIK3R1* no amplification	(211, 212)
PIK3C2A *PIK3C2B*		no information		amplification	(211)
PTEN		no mutations	(216)	frequent genetic alterations (50%-70%)	(103, 106, 107)

Table 5 Genetic alterations pertaining to components of the PI3K signaling pathway in NB and GB. Most studies are aimed at investigating chromosomal aberrations of *PIK3CA*, while the mutational status of the other isoforms remains largely unknown.

2.5.2.2 Receptor Tyrosine Kinases

RTKs play a crucial role in the biology of many types of human cancer (161). In principle, any receptor with tyrosine kinase activity possesses oncogenic potential. Different mechanisms can lead to aberrant RTK activation and ultimately to aberrant downstream signaling. These alterations are best exemplified by the EGFR, which has been reported to be involved in the pathogenesis of a variety of human malignancies, including brain, breast, lung and prostate cancer (218). Malignant transformation by oncogenic EGFRs can occur through a number of mechanisms including: (i) receptor over-expression (108), (ii) deletion mutations, resulting in ligand-independent RTK activation (113, 114), (iii) mutations that affect the dimerization properties, leading e.g. to constitutive receptor dimerization (219), (iv) activation of autocrine growth factor loops (115, 116) and finally, (v) altered endocytosis of activated receptors, leading to prolonged signaling (220). These mechanisms have also been shown to be involved in aberrant signaling emanating from other RTKs, including the IGF-IR or the PDGFR. Besides providing the cell with a survival advantage, constitutively active RTKs can have a chemoprotective effect, making treatment of patients very difficult (221).

Neuroblastoma In NB, various growth factors have been identified that play a key role in tumor cell proliferation as well as in chemoresistance. Early reports demonstrated autocrine signaling loops involving IGF-II (222) and stem cell factor (SCF) (223). More recently, it was shown that the hepatocyte growth factor (HGF)/c-Met pathway contributes to NB invasiveness and malignant transformation (224). The role of EGF and PDGF signaling remains ambiguous. More than a decade ago, expression of different PDGFR chains was reported in NB cell lines (225). Although it has since been shown that expression of the corresponding ligand (PDGF) correlates with advance-stage disease (226), the contribution of this growth factor system remains unclear. The role of EGF signaling has not been studied extensively, despite a recent report demonstrating that proliferation of human NB is mediated by the EGFR (227). By contrast, the Trk-family receptors, which are normally involved in regulating basic cellular functions in neuronal cells, have been the focus of much attention in NB research (228). Interestingly, over-expression of individual members was found to correlate with clinical behavior. While TrkA expression is characteristic of favorable tumors (229), TrkB expression is associated with unfavorable, *MYCN*-amplified NB (230).

Glioblastoma The list of growth factors implicated in the biology of malignant glioma is long (128). In addition to the intensely studied EGFR and PDGFR (discussed in sections 2.2.3.3. and 2.2.3.4, respectively) a number of other RTKs are involved in gliomagenesis. Expression of IGFs and their corresponding receptors has been extensively studied and has led to the proposition of an autocrine signaling loop (123, 231, 232). Furthermore, the HGF/c-Met pathway has also been found to be deregulated in glioma and appears to be involved in the transition from low to high grade tumors (233, 234). Considering the fact that gliomas are highly vascularized tumors, it is not surprising that aberrant signaling involving vascular endothelial growth factor (VEGF) has been reported in gliomas. In particular, expression of this growth factor seems to be involved in the transition to high grade tumors (235, 236).

In summary, various growth factor systems are implicated in the biology of NB and GB and a plethora of studies have substantiated the essential contribution of aberrant RTK signaling to tumor development and/or progression in numerous human cancers.

2.5.2.3 Other Signaling Components

A variety of alterations have been associated with proteins known to contribute substantially to PI3K signaling. Mutational analysis has demonstrated that downstream molecules, such as Akt and PDK1 are subject to genetic changes in human cancer, albeit at low rates (237-241). More recently, extensive gene sequencing analysis has provided a plethora of information concerning the mutational status of numerous signaling regulators (241-244). Although these studies revealed a number of previously uncharacterized genetic alterations, no recurrent novel mutations were detected. In recent years, tissue microarray analysis has allowed visualization of pathway activation in a large number of primary tumor specimens. This has provided a better understanding of the effects genetic alterations have on signaling pathway activation and, in some cases, has revealed novel prognostic markers (245-247).

Neuroblastoma To date, little is known about the mutational status of genes involved in PI3K signaling in NB. In an attempt to find classifiers predicting patient outcome, large-scale cDNA microarray analysis of tumor samples has been performed by a number of groups (3, 4). The only PI3K pathway component found be relevant for distinguishing between patient groups was altered expression of *PIK3R1* (4). However, a recent tissue microarray study found that phosphorylation of Akt and the ribosomal S6 protein is frequently elevated in primary NB (248). Moreover, phosphorylation of Akt was found to correlate with aggressive

disease and *MYCN* amplification and could be shown to be a novel prognostic indicator of poor outcome in NB (248). Future studies will hopefully complement these initial findings, thus leading to a better understanding of the mechanisms contributing to aberrant PI3K signaling in NB.

Glioblastoma Mutational analysis of genes involved in PI3K signaling revealed occasional amplification of the gene encoding Akt, while the *FRAP1* gene, which encodes mTOR, was not found to be amplified when compared with non-neoplastic brain tissue (211). Furthermore, no amplification of the gene encoding S6K was detected in GB (211). A tissue microarray study demonstrated that loss of *PTEN* is highly correlated with Akt activation (95). Furthermore, a significant correlation was observed between activation of Akt and phosphorylation of downstream signaling molecules, including the S6 protein. A more recent study confirmed the correlation between activation of Akt and S6 phosphorylation (96). In addition, activation of TSC2, which is thought to be the main downstream mediator of Akt in mTOR activation, was found to significantly correlate with Akt activation. A study aimed at examining the prognostic value of PI3K pathway activation revealed a significant association between PI3K, Akt and S6K phosphorylation and increasing tumor grade, decreased levels of apoptosis and adverse clinical outcome (247). In summary, these results highlight the pivotal role aberrant PI3K signaling plays in glioma biology.

2.5.3 Targeting the PI3K Pathway in Human Cancer

2.5.3.1 Rationale for Targeting Tyrosine Kinases

A prominent feature of many tumors involves aberrant signaling involving a number of tyrosine kinases. Therefore, the use of small molecule inhibitors targeting the kinase activity of these signaling components appears to show much promise. The observation that human cancers frequently display over-expression or mutation of tyrosine kinases, including RTKs, has led to the development of small molecule inhibitors that specifically target these proteins. The most renowned examples of specific tyrosine kinase inhibitors include Gleevec (Imatinib mesylate) and Herceptin (Trastuzamab). These two inhibitors exemplify the principal mechanisms of targeted therapy: pharmacological inhibition of tyrosine kinase activity and blocking of receptor function through monoclonal antibodies.

Gleevec is a small molecule inhibitor which was identified in a screen aimed at identifying compounds capable of inhibiting the ABL kinase (249). This search was initiated by the observation that the majority of patients with chronic myelogenous leukemia (CML) harbor a characteristic translocation, known as the Philadelphia chromosome, which encodes the fusion protein Bcr-Abl (250). Expression of this fusion protein leads to aberrant intracellular signaling, ultimately leading to the massive clonal expansion of hematopoeitic stem cells (250). Based on the fact that Bcr-Abl can be detected in nearly all patients with CML, this oncogene is considered an ideal target. Indeed, Gleevec has shown striking results in a high percentage of CML patients (251). This compound has since been integrated into the standard treatment regimen of these patients. Due to the inhibitory effect Gleevec has on other tyrosine kinases, including the c-kit receptor and the PDGFR, its success is not limited to CML. Striking clinical responses have been observed in a subset of gastrointestinal stromal tumor patients which display a mutant form of the c-kit receptor, thus expanding the repertoire of this compound to other cancers.

Herceptin is a humanized monoclonal antibody designed to target the Her2 receptor, thereby inhibiting growth of Her2-overexpressing cells (252). The rationale for the development of this molecule was the observation that Her2 is amplified in roughly a third of all patients with invasive breast cancer. This molecular marker is easily detected by immunohistochemical analysis, thus allowing selection of patients most likely to respond to treatment with Herceptin. Promising clinical responses have allowed the integration of this antibody into standard treatment protocols (253).

While the effects of Herceptin are of great importance to patients with breast cancer, there are limits to the success of antibody-mediated targeting of tyrosine kinases. Numerous mechanisms of resistance to therapy are known, including loss of *PTEN* or the presence of circulating truncated forms of Her2, which lead to decreased levels of antibodies available for binding of membrane-anchored Her2 (254, 255). Moreover, their range of action is restricted to the cell surface and depends on an intact extracellular domain. Small-molecule inhibitors can cross the cell membrane, thus allowing access to a larger number of targets. These compounds occupy the ATP binding site of catalytic domains, thereby directly preventing protein activation. In recent years, a plethora of new kinase inhibitors has evolved from large scale screenings with compound libraries (256, 257). While the success story of Gleevec raised much hope, it seems to be an exception in the field of drug development. There are different reasons for this daunting realization. The majority of human malignancies are genetically complex, and it is unlikely that their survival solely depends on the activation of a single molecule or RTK (256, 257). Moreover, advanced tumors are genetically unstable, allowing the emergence of secondary mutations within the kinase domains of proteins, thus potentially leading to acquired resistance to pharmacological inhibitors (258). While targeted strategies rarely equal the status of first-line treatment, their use as adjuvants has proven efficacious (259). Although it is unlikely that newly developed inhibitors will be able to keep pace with Gleevec, the availability of a growing number of compounds could allow increasingly targeted therapies, tailored to the molecular fingerprint of individual tumors.

2.5.3.2 Rationale for Targeting the PI3K Pathway

Considering that certain human cancers, including NB and GB, are found to express a variety of different RTKs, it remains unclear whether targeting individual receptors will provide a successful therapeutic strategy. An alternative approach would be to identify downstream signaling molecules essential for transmitting the proliferative and survival message of several different RTKs. The family of PI3K represents such a molecule, in view of their crucial role in controlling cell proliferation, survival and motility/metastasis downstream of many different RTKs (142, 144, 260). Furthermore, a growing body of evidence suggests that that activation of the PI3K/Akt signaling pathway plays an important role in conferring broad-spectrum chemoresistance to cancer cells (261-264). Thus, concomitant inhibition of the PI3K/Akt pathway could sensitize tumor cells to chemotherapeutic agents, enhancing cytotoxicity.

Neuroblastoma Activation of the PI3K/Akt pathway has been shown to determine the sensitivity of NB cells to chemotherapeutic agents and targeted RTK inhibition *in vitro* (265-268). This is exemplified by the finding that the BDNF/TrkB system contributes to chemoresistance of NB cells via the PI3K/Akt pathway (266). Further investigations identified Akt as a key regulator and showed that pharmacological inhibition of Akt sensitized NB cells to chemotherapy (267). Our own work demonstrated that cells with increased phosphorylation of Akt and the S6 protein were more resistant to NVP-AEW541, an IGF-IR inhibitor (Paper 3.2.2; (265)). Inhibition of Akt activity restored the sensitivity to NVP-AEW541, corroborating the contribution of Akt to chemoresistance (265). Moreover, it was observed that drug-resistant cells secrete factors capable of activating the PI3K/Akt pathway in drug-sensitive cells, thus attenuating their response to doxorubicin (268). Although the exact mechanism remains elusive, this finding further highlights the importance of the PI3K/Akt pathway in regulating the sensitivity of NB cells to chemotherapeutic agents. Interestingly, it was recently reported that treatment of *MYCN*-amplified NB cell lines with LY294002, a broad-spectrum PI3K inhibitor, results in decreased N-Myc protein expression levels, while mRNA levels remain unaltered (269). A subsequent study showed that treatment of NB cells with rapamycin (an mTOR inhibitor) also led to reduced protein expression levels of N-Myc (270). These observations illustrate the complexity of PI3K signaling and certainly merit further investigation. While a recent analysis showed a correlation between activation of Akt and poor prognosis (248), no patient-based study has investigated the relationship between PI3K/Akt pathway activation and response to chemotherapeutic agents.

Glioblastoma Considering that genetic alterations involving *PTEN* are frequent events in GB and are associated with elevated Akt activity (97), it is not surprising that the PI3K/Akt pathway has been found to contribute substantially to chemosensitivity. Loss of *PTEN* makes it particularly difficult to target RTKs, because receptor inhibition becomes uncoupled from downstream signaling pathways. In contrast to NB, a number of studies have tried to directly assess the link between activated signaling components and the susceptibility to drug-induced cell death in primary gliomas, thus allowing the identification of patients most likely to respond to treatment. Expression of the EGFR and downstream signaling components was analyzed in the context of response to the EGFR inhibitor erlotinib (271). This study found that tumors with high EGFR expression and low levels of phosphorylated Akt had a better response to erlotinib than those with low EGFR expression and high levels of phosphorylated Akt (271). A study published shortly thereafter reported that co-expression of PTEN and the

mutated EGFRvIII significantly correlated with responsiveness to EGFR kinase inhibitors (272). These and other studies have convincingly demonstrated that deregulation of Akt activation contributes to chemoresistance in GB.

Numerous studies have indicated that Akt is a key mediator of chemosensitivity and therefore the feasibility of targeting this molecule has been widely discussed (273, 274). However, it is important to keep in mind that Akt represents a crucial nodal point in PI3K signaling, integrating extracellular signals and orchestrating subsequent pathway activation. Therefore, general inhibition of this molecule could potentially be accompanied by serious side effects. Targeting the PI3K family could offer a practicable alternative considering their pivotal role in relaying activating signals from a variety of different RTKs. Although the classical paradigm suggests p85/p110α as the main PI3K complex involved in signal transduction, the existence of eight isoforms leaves room for speculation. In addition, the observation that these proteins are expressed in a tissue-specific manner and seem to have non-redundant functions makes them interesting targets (188, 189, 275-277). While a vast number of compounds has been developed to inhibit various components of the PI3K/Akt signaling pathway, the following discussion is limited to PI3K inhibitors.

LY294002 and Wortmannin

The best characterized PI3K inhibitors are LY294002 and Wortmannin, which both display activity against multiple PI3Ks. These compounds have been shown to effectively inhibit *in vitro* and *in vivo* growth of a variety of cancer cells (278-280). Furthermore, when used at low doses, they have been shown to enhance the cytotoxic effect of many chemotherapeutic agents (261, 281, 282). In addition, a radiosensitizing effect has been observed in cells treated either with LY294002 or Wortmannin (283, 284). Despite these promising observations, neither compound has entered clinical trials due to high toxicity. Nevertheless, they have proved indispensable in dissecting the PI3K/Akt signaling pathway and providing insight into the mechanisms leading to chemoresistance.

Isoform-Specific PI3K Inhibitors

In recent years, numerous attempts have been made to develop isoform-specific PI3K inhibitors, in order to circumvent the toxic effects of inhibiting multiple PI3K isoforms (188, 189, 275-277). These efforts have indeed led to the generation of compounds that exhibit increased specificity towards individual isoforms. However, their clinical feasibility remains to be investigated. In the following paragraphs, inhibitors of the class I_A PI3Ks are discussed.

p110α Screening of a chemical library led to the identification of some of the first compounds described to selectively inhibit p110α (285). The systematic search for specific PI3K inhibitors triggered the development of numerous other compounds with increased specificity towards p110α (286, 287) and led to the discovery of compounds which selectively target other catalytic isoforms, including p110β (288). Another p110α inhibitor, termed PI-103, was recently identified in a chemical array because of its unique activity against genetically diverse glioma cell lines (289). The striking activity of this compound was traced to its ability to selectively block not only p110α, but also mTOR (289). This compound was found to be well-tolerated *in vivo* and was highly effective against glioma xenografts (289). However, a subsequent study found that PI-103 inhibits all class I catalytic PI3K isoforms in the low nanomolar range and is therefore not specific for p110α (290). Nevertheless, this molecule revealed the feasibility of targeting multiple molecules within the same pathway, thereby inhibiting feedback-mechanisms that can lead to pathway activation as observed with the feedback-loop involving mTOR and Akt (291).

p110β A p110β-specific inhibitor termed TGX-221 was developed in an attempt to understand the contribution of p110β to platelet activation (292) and is now commercially available.

p110δ The lack of isoform-specific inhibitors also prompted the development of a p110δ inhibitor, termed IC87114. This inhibitor was studied in neutrophils in order to investigate the contribution of p110δ to chemotaxis (293). IC87114 has been most extensively studied in cells of the hematopoeitic system and was found to affect proliferation and chemosensitivity in acute myeloid leukemic cells, while normal hematopoeitic progenitor cells remained unaffected (294).

The availability of novel small molecule inhibitors of individual PI3K isoforms is very exciting. Not only do they provide a valuable tool to elucidate isoform-specific roles in cellular responses, but they also raise hope of being able to selectively target this fundamental survival pathway in cancer cells with minimal side-effects. Preclinical trials have demonstrated that pharmacological inhibition of individual PI3K isoforms results in impaired proliferation of a variety of human cancer cells (188, 189, 275-277). While it is highly unlikely that the use of PI3K-specific inhibitors as single agents will ever be feasible, their combined use with other agents could have a synergistic effect and is thus a promising area of application (188, 189, 275-277). However, a number of important issues remain to be addressed. Considering the complexity of PI3K signaling, which involves cross-talk with other pathways and internal feedback-loops, a detailed understanding of this cascade is an absolute must in order to prevent unwanted side-effects. A further challenge arises from the high sequence homology within the catalytic domain of class I_A PI3Ks, which has impeded the development of truly isoform-specific inhibitors. Moreover, a number of allegedly specific inhibitors have been found to show activity against PI3K-like kinases, including ATM or DNA-PK, thus limiting their use. It must also be kept in mind that the existence of isoform-specific PI3K inhibitors will not be beneficial for patients until molecular markers and genetic fingerprints have been defined which allow the identification of tumors most likely to respond to treatment. A further challenge to targeted therapies is the fact that most tumors, especially those in advanced stages, do not rely on the activation of a single pathway for survival (259). Working out the ideal combination of proteins to inhibit simultaneously should contribute to the success rate of future targeted therapies. Despite these unresolved issues, PI3Ks remain interesting molecules for targeted therapeutic approaches (188, 189, 275-277). Increased activation of this pathway in human cancer is well documented and is achieved by a number of genetic alterations which affect the signaling cascade at different levels. The existence of eight PI3K isoforms necessitates a detailed understanding of the individual functions, thus delaying the race to develop inhibitors for clinical use. Nevertheless, the wait could well pay off, considering that these proteins are expressed in a tissue-specific manner and seem to have non-redundant functions, thus making them very attractive targets

3 Results

3.1 Project Descriptions

3.1.1 Targeting the phosphoinositide 3-kinase isoform p110δ impairs growth and survival in neuroblastoma cells *(Clinical Cancer Research, in press)*

NB is a childhood neoplasm arising from neural crest cells. According to the NCI, NB accounts for 7-10% of all cancers diagnosed in children younger than 15 years of age. The aim of this study was to assess the contribution of individual PI3K isoforms to the survival of NB cells. Cell lines and primary tumors were analyzed for PI3K expression both at the mRNA and protein level. Based on the observation that p110δ is highly over-expressed in primary tumors, cell lines were generated in which p110α or p110δ were stably downregulated using RNA interference (RNAi). Cellular responses, such as basal proliferation and PI3K/Akt/mTOR pathway activation upon growth factor stimulation were then compared. While downregulation of either p110α or p110δ led to impaired cell growth, reduced expression of p110δ had a selective effect on the survival of SH-SY5Y cells. Decreased levels of p110δ were found to induce apoptosis and lead to lower expression levels of anti-apoptotic Bcl-2 family proteins. SH-SY5Y cells with decreased p110δ levels also displayed reduced activation of S6K in response to stimulation with EGF or IGF-I. These studies unveiled a novel role for p110δ in the regulation of NB survival, and demonstrated that p110α and p110δ have non-redundant functions in these tumor cells.

I performed all experiments aside from Figures 1B (contributed by Alexander Schramm) and 5B (performed by Kathrin Doepfner).

3.1.2 Protein kinase B modulates the sensitivity of human neuroblastoma cells to insulin-like growth factor inhibition *(International Journal of Cancer)*

IGF signaling has been extensively studied in the context of NB proliferation, survival and motility and several potential therapeutic approaches involving this growth factor system have been reported. This study was aimed at evaluating the potential of a novel IGF-IR inhibitor (NVP-AEW541, Novartis) in NB. Moreover, the effect of concomitant inhibition of PI3K/Akt signaling components was assessed. NVP-AEW541 was found to inhibit NB cell proliferation with varying IC_{50}-values. An inverse correlation was observed between levels of phosphorylated Akt and S6 protein and sensitivity to the inhibitor. Treatment of cells with an Akt inhibitor restored sensitivity to the IGF-IR inhibitor. In addition to its anti-proliferative effects, NVP-AEW541 was found to sensitize NB cells to cisplatin-induced apoptosis. These results underscore the importance of IGF signaling in NB and highlight the potential of targeting the IGF-IR as a novel therapeutic approach in this pediatric cancer.

In this manuscript, I performed the experiments with the DNAkt contruct (in Figure 8).

3.1.3 Novel role for insulin as an autocrine growth factor for malignant brain tumor cells *(Biochemical Journal)*

Atypical teratoid/rhabdoid tumors (AT/RTs) of the CNS are childhood malignancies associated with poor survival rates due to resistance to conventional treatments such as chemotherapy. The present study was aimed at gaining a more profound insight into the involvement of various RTKs in the survival of AT/RT cells. Both the insulin receptor (IR) and the IGF-IR were found to be over-expressed when compared to normal human brain tissue. Furthermore, secretion of IGF-I was detected, suggesting the existence of an autocrine signaling loop in these cells. IGF-I was shown to effectively activate Akt, indicating a crucial involvement of the PI3K/Akt signaling pathway in the signaling loop. By means of isoform-specific PI3K inhibitors and RNAi, the p110α isoform was identified as a key mediator of AT/RT survival and signaling from the activated IGF-IR. Together, these results reveal a novel role for autocrine signaling by insulin and the IR in the growth and survival of malignant AT/RT cells via the PI3K/Akt pathway.

My contribution to this manuscript involved ELISA mesurements of IGF-I and IGF-II in samples .

3.1.4 Targeting PI3KC2β impairs proliferation and survival in acute myeloid leukemia, brain tumors and neuroendocrine tumors *(submitted to the British Journal of Cancer)*

While much attention has been given to the class I PI3K isoforms, little is known about the functions of class II PI3Ks in human cancer. This study was aimed at investigating the role of the PI3KC2β isoform in a panel of primary tumors and cell lines. Over-expression of PI3KC2β was detected in subsets of tumors and cell lines from acute myeloid leukemia (AML), GB, medulloblastoma, NB, and small cell lung cancer (SCLC). Specific pharmacological inhibitors of PI3KC2β or small interfering RNA (siRNA) impaired proliferation of a panel of cell lines and primary cultures from AML, brain tumors and neuroendocrine tumors. Inhibition of PI3KC2β also induced apoptosis in AML and GB cell lines and sensitised the cells to chemotherapeutic agents. Furthermore, PI3KC2β inhibition impaired the phosphorylation of downstream signalling mediators in AML. Together, these data show that PI3KC2β contributes to proliferation and survival in AML, brain tumors and neuroendocrine tumors and may represent a novel target in these malignancies.

In this manuscript, I performed all experiments involving NB and GB. Together with Kathrin Doepfner, I was responsible for drafting and formatting this manuscript.

3.1.5 Distinct class IA PI3K isoforms regulate glioblastoma cell growth, survival and migration *(Manuscript in preparation)*

GB is a common malignant form of brain tumor associated with poor prognosis. The PI3K/Akt pathway is frequently activated in GB due to mutations in *PTEN*, *PIK3CA* or due to mutation or over-expression of RTKs. The expression and functions of the catalytic class I_A PI3K isoforms were investigated in GB cell lines and *ex vivo* cultures. Targeting the isoforms p110β or p110δ using RNAi or isoform-specific inhibitors did not significantly impair the growth of GB cells under normal culture conditions, while inhibition of p110α had a partial effect. However, the isoforms p110α and p110β were found to contribute to GB growth under anchorage-independent conditions. We further observed a selective role for p110β for protecting GB cells from anoikis (detachment-induced apoptosis). In addition, p110β was essential for the ability of GB cells to migrate, while p110α and p110δ were dispensable for the response. The p110α isoform also appeared to play a role in the chemosensitivity of GB

cells towards doxorubicin and cisplatin. Moreover, p110α is essential for RTK coupling to the downstream effectors Akt and S6K. Together, these results uncover distinct functions for the catalytic class I_A PI3K isoforms p110α and p110β in GB cell responses.

In this manuscript, I performed all experiments except those depicted in Figure 3B-D, which were performed by Olivier Pardo.

3.2 Manuscripts

3.2.1 Targeting the phosphoinositide 3-kinase isoform p110δ impairs growth and survival in neuroblastoma cells

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Running title: Role of p110δ in neuroblastoma

Keywords: neuroblastoma; phosphoinositide 3-kinase; Akt; mTOR; S6K

2

Abstract

Purpose: The phosphoinositide 3-kinase (PI3K)/Akt pathway is frequently activated in human cancer and plays a crucial role in neuroblastoma biology. We were interested in gaining further insight into the potential of targeting PI3K/Akt signalling as a novel anti-proliferative approach in neuroblastoma.

Experimental Design: The expression pattern and functions of class I_A PI3K isoforms were investigated in tumor samples and cell lines. Effects on cell survival and downstream signalling were analysed following down-regulation of p110α or p110δ in SH-SY5Y and LA-N-1 cells by means of RNA interference (RNAi).

Results: Over-expression of the catalytic p110δ and regulatory p85α isoforms was detected in a panel of primary neuroblastoma samples and cell lines, as compared to normal adrenal gland tissue. While down-regulation of either p110α or p110δ led to impaired cell growth, reduced expression of p110δ also had a selective effect on the survival of SH-SY5Y cells. Decreased levels of p110δ were found to induce apoptosis and lead to lower expression levels of anti-apoptotic Bcl-2 family proteins. SH-SY5Y cells with decreased p110δ levels also displayed reduced activation of ribosomal protein S6 kinase (S6K) in response to stimulation with epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1).

Conclusions: Together, our data reveal a novel function of p110δ in neuroblastoma growth and survival.

3

Introduction

Neuroblastoma is the most common extra cranial solid tumor occurring in children and accounts for 8% to 10% of all paediatric malignancies (1, 2). High-risk disease, present in about half of the patients, is characterised by unresectable primary lesions and metastasis (2). Treatment of high-risk neuroblastoma with high-dose chemotherapy including peripheral stem cell rescue and radiotherapy resulted only in 34% three-year event-free survival probability (3).

A better understanding of the biology of neuroblastoma will potentially lead to the identification of novel therapeutic targets, which in turn could facilitate the development of new drugs for neuroblastoma. A promising field of investigation is to target receptor tyrosine kinase (RTK) signalling to some of their downstream mediators such as phosphoinositide 3-kinase (PI3K), Akt and the mammalian target of rapamycin (mTOR). Polypeptide growth factors have indeed been shown to play a key role in neuroblastoma biology. Insulin-like growth factor (IGF) signalling has been extensively studied in the context of neuroblastoma proliferation, survival and motility (4-7). Several potential anti-tumor approaches involving the IGF-1 system have been reported in neuroblastoma (8, 9). Moreover, inhibition of platelet-derived growth factor receptor (PDGFR) and c-Kit signalling with imatinib mesylate was reported to impair growth in neuroblastoma cell lines (10). Neurotrophins such as brain-derived neurotrophic factor (BDNF) also play an important role in neuroblastoma chemoresistance by binding to the Trk receptor family (11).

In view of the fact that neuroblastoma express a variety of different RTKs, it remains unclear whether targeting individual receptors will provide a successful therapeutic strategy. An alternative approach would be to identify downstream signalling molecules essential for transmitting the proliferative and survival message of several different RTKs. Phosphoinositide 3-kinase (PI3K) represents such a molecule, in view of its crucial role in controlling cell proliferation, survival and motility/metastasis downstream of many different RTKs (12-14). PI3Ks are an enzyme family comprising eight catalytic isoforms in humans, with different substrate specificities, regulatory mechanisms and tissue distribution (12, 14). The importance of PI3K signalling in human cancer was first demonstrated by the observation that mutations in the tumor suppressor gene *PTEN* occur frequently in human tumours. PTEN is a phosphatase that antagonises the action of PI3K by dephosphorylating the D-3 position of polyphosphoinositides (14, 15). Moreover, recent reports have described activating mutations in the *PIK3CA* gene encoding the catalytic p110α isoform of PI3K in a variety of human cancers, including breast, colon and ovarian cancer, as well as medulloblastoma (16). In neuroblastoma, brain-derived neurotrophic factor (BDNF) was shown to protect the tumor cells from chemotherapy-induced apoptosis via the PI3K pathway (17). IGF-1 signalling via PI3K was shown to be required for neuroblastoma differentiation, cytoskeletal rearrangements (18), as well as angiogenesis and vascular endothelial growth factor (VEGF) expression (19). PI3K signalling is also activated by epidermal growth factor (EGF) in neuroblastoma cells and contributes to cell

proliferation by this growth factor (20). Thus, targeting the PI3K/Akt/mTOR/S6K pathway may represent an attractive novel approach to develop therapies for neuroblastoma.

In the present report we have evaluated the expression of PI3K isoforms in primary human neuroblastoma samples and cell lines. Moreover, we have investigated whether targeting distinct PI3K isoforms could impair growth and survival of neuroblastoma cell lines. Our results show for the first time that the class I_A PI3K p110δ is over-expressed in a subset of neuroblastoma samples and plays a crucial role in the growth and survival of neuroblastoma cells.

Materials and methods

Reagents and Antibodies. Antibodies were purchased from the following companies: p85α, p110β, p110δ, PARP, PTEN, Akt/PKB, Erk1/2, Santa Cruz Biotechnology; S6 protein, 4EBP1 and phosphospecific antibodies for Akt/PKB (Ser473; Thr308), Erk1/2 (Thr202/Tyr204), S6 protein (Ser235/236; Ser240/244), 4E-BP1 (Thr37/46), Cell Signaling Technology; β-Actin, Sigma-Aldrich; p110α (clone U3A), generous gift from Dr. A. Klippel. Analysis of proteins involved in apoptosis was performed using the Pro-Survival and the Pro-Apoptosis Bcl-2 Family Antibody Sampler Kit (Cell Signaling). LY294002, rapamycin, EGF, PDGF and IGF-1 were obtained from Calbiochem.

Determination of PI3K-related gene expression in human primary neuroblastoma samples by cDNA microarray analysis. Expression of PI3K-related genes was determined in tumours from 68 neuroblastoma patients by Affymetrix U95A array analysis. The patient cohort and data normalisation procedures have been described elsewhere (21). Correlation of PI3K-related gene expression with molecular and clinical parameters was determined using the stats package included in R2.2 (www.r-project.org). Visualisation of gene expression was accomplished using Spotfire 8.1.

Primary neuroblastoma samples. Ethical approval to use residual tissue was obtained. RNAlater-preserved tumor tissue was available from the Swiss Paediatric Oncology Group tumor bank from neuroblastoma patients diagnosed between January 2003 and December 2005 at the University Children's Hospital of Zurich, (n=14), the Children's Hospital Luzern (n=3), the University Children's Hospital Bern (n=1) and the University Children's Hospital Basel (n=1). The selection of the tumours for the study was based on the availability of a sufficient quantity of tumor tissue to perform RNA isolation. All diagnoses were confirmed by histological assessment of the tumor specimen obtained at surgery. An overview of the tumor characteristics is given in Supplemental Fig. 1.

Protein extraction from tumor samples. Tumor tissue was disrupted with a sterile disposable tissue grinder (Sage Products Inc.). Protein extracts were obtained using the PARIS Kit (Ambion) according to the manufacturer's instructions.

Isolation of RNA from tumor samples and RT-PCR. Tumor tissue was disrupted as described above and homogenised in guanidinium isothiocyanate-containing buffer. Total RNA was isolated using the RNeasy kit (Qiagen Inc.) according to the manufacturer's protocol. Total RNA (3 μg) from each tumor sample was converted into cDNA using the SuperScript™ First-Strand Synthesis System for PCR according to manufacturer's instructions (Invitrogen Life Technologies). mRNA expression of four target genes and 18S (internal control gene) was measured in tumor samples and cell lines by TaqMan® Assay-on-Demand™ Gene Expression products (Applied Biosystems). Normal human adrenal gland tissue (AG) was used as a reference. The following primers were used (gene – assay ID): PIK3R1 - Hs00236128_m1; PIK3CA - Hs00180679_m1; PIK3CB - Hs00178872_m1;

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PIK3CD - Hs00192399_m1; eukaryotic 18S rRNA - Hs99999901_s1. Three replicates were run for each sample in a 96-well format plate. Gene expression assays consisted of a FAM™ dye-labelled TaqMan® MGB probe and two PCR primers. The thermal cycling conditions consisted of an initial denaturation step at 95°C for 10 min and a 50-cycle countdown at 95°C for 15 s and 60°C for 1 min. Each sample was normalised on the basis of its 18S rRNA content. Relative mRNA expression levels were calculated using the comparative threshold cycle (CT) method (22).

Cell Culture. Human neuroblastoma cell lines were kindly provided by Dr Brodeur, Children's Hospital of Philadelphia. The cells were grown in RPMI (Life Technologies/Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin/streptomycin/L-glutamine and passaged every 3-5 days by trypsinization.

Stable Transfectants. SH-SY5Y and LA-N-1 cells were stably transfected with the murine ecotrophic receptor (EcoR) using Lipofectamine (Invitrogen) according to the manufacturer's protocol. 72 hours post transfection, cells were diluted in medium containing G418 (0.8 mg/ml). In parallel, retroviral plasmid constructs encoding short hairpin RNA (shRNA) specifically targeting p110α or p110δ (23) were transiently transfected into packing cells. The supernatant was diluted with 1 part RPMI containing G418 (final concentration: 0.8 mg/ml), as well as Polybrene (final concentration: 8μg/ml). The diluted supernatant was then added to cells stably expressing the EcoR. 24 hrs post infection, SH-SY5Y and LA-N-1 cells were split in medium containing puromycin (0.5 μg/ml). Single colonies were picked and expanded in selective medium. Protein down-regulation was confirmed by Western blot analysis. All experiments were performed with two clones from each transfection.

Transient Transfection. Cells were transiently transfected using the Amaxa Nucleofector Device according to the optimised protocol provided for SH-SY5Y cells. The following constructs were used: pRS, pRS-PIK3CA, pRS-PIK3CD (23), pcDNA3 (Invitrogen) and pcDNA-S6K AK (9).

Cell Proliferation. Neuroblastoma cells were seeded in 96-well plates at a density of 7'500 cells/well and grown for 72 hrs in RPMI containing low (1%) or high (10%) serum. Alternatively, cells were treated with growth factors or inhibitors as indicated. Cell proliferation was analysed by the CellTiter 96® AQ_{ueous} Cell Proliferation Assay (Promega) according to the manufacturer's instructions.

Apoptosis. Neuroblastoma cells were seeded in 96-well plates at a density of 20'000 cells/well. Basal caspase-3/7 activity was measured after 24 hrs using the Caspase-Glo® 3/7 Assay (Promega) according to the manufacturer's instructions.

Cell Cycle Distribution. Cell cycle distribution was analysed by means of propidium iodide (PI) staining and Fluorescence-activated cell sorter (FACS) analysis. Cells were seeded in a 6-well plate at a density of 6.5 x 10^5 cells and incubated in RPMI containing low (1%) or high (10%) serum for 24 hrs. Cells were collected using 0.5% trypsin and resuspended in cold PBS. One-tenth volume of

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10x propidium iodide solution (500 μg/ml propidium iodide, 10mg/ml sodium citrate and 1% (v/v) Triton X-100) was added and cells were acquired in a flow cytometer within half an hour.

Growth Factor Stimulations. Cells were grown to confluency in a 6-well plate and starved overnight in RPMI containing 0.5% FCS. Cells were maintained in serum-free RPMI for 1 hour in the presence or absence of inhibitors as indicated and were then stimulated with the indicated growth factors for 10min. Cellular lysates were prepared as described below.

Western blotting. Cellular lysates were prepared as described (24) and normalised using a bicinchoninic acid (BCA) protein assay (Pierce). Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Biosciences) and immunoblotted with the indicated antibodies prior to chemiluminescent detection (ECL Western blotting detection reagents; Amersham Biosciences).

Statistical Analysis. Spearman's rank correlation and the Exact Wilcoxon rank-sum test were used for the analysis of PI3K gene expression in primary neuroblastoma samples. For experiments on cell lines, the statistical significance of differences between groups was assessed with ANOVA using the Bonferroni multiple comparison test, $p < 0.01$ were indicated with a double asterisk.

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Results

Over-expression of PI3K isoforms in primary human neuroblastoma samples and cell lines. To gain insight into the specific functions of class I_A PI3K isoforms in neuroblastoma, we initially assessed the expression levels of the regulatory and catalytic subunits in two independent groups of tumor samples. In a panel of 19 primary neuroblastoma samples, the expression levels of the class I_A PI3K regulatory and catalytic subunits were analysed by quantitative RT-PCR (qRT-PCR) (Fig. 1A). Expression of the *PIK3R1* (encoding the regulatory subunit p85α) and *PIK3CD* (encoding the catalytic subunit p110δ) genes was found to be increased more than 2-fold in 10/19 neuroblastoma samples when compared to normal adrenal gland tissue (Fig. 1A, left panel). In contrast, mRNA expression of the catalytic p110α and p110β isoforms was not increased (Fig. 1A, left panel). In this panel of tumor samples, expression of p85α and p110δ was significantly correlated (Spearman's rank correlation, $p<0.0001$), indicating that the p85α/p110δ heterodimer is over-expressed in neuroblastoma. Expression of p85α and p110δ was significantly higher in children under the age of one (median values: 2.48 for p85α and 5.17 for p110δ) than in patients older than one year of age (median values: 0.64 for p85α and 0.95 for p110δ) (Exact Wilcoxon rank-sum test, $p=0.0121$ for p85α; $p=0.0025$ for p110δ). Furthermore, the expression of p85α and p110δ was found to be significantly lower in neuroblastoma samples with *MYCN* amplification (median values: 2.42 for p85α and 4.63 for p110δ) than without amplification (median values: 0.81 for p85α and 0.75 for p110δ) (Exact Wilcoxon rank-sum test, $p=0.0339$ for p85α; $p=0.0339$ for p110δ). In contrast, no correlation was found between p85α and p110δ expression and tumor stage, 1p status or progression. Thus, p85α and p110δ expression was found to be increased in neuroblastoma samples from children under the age of one and with no *MYCN* amplification.

To investigate if *PIK3CD* mRNA levels are predictive of protein expression levels, Western blot analysis was performed on twelve of the patient samples. In 10/12 samples, mRNA levels correlated with protein expression (Fig 1A, right panel).

Reanalysis of cDNA microarray data of an independent panel of 68 primary neuroblastoma samples also revealed striking variations in the expression levels of the class I_A PI3K regulatory subunit p85α and the catalytic subunit p110δ. Expression of the p85α and p110δ subunits was significantly higher in samples from children under the age of one compared to patients older than one year of age (Fig. 1B), confirming the results of the qRT-PCR analysis (Fig. 1A).

We next investigated the expression of PI3K isoforms in a set of 8 representative neuroblastoma cell lines to validate the findings obtained in tumor samples. In this panel of cell lines, p85α was over-expressed in 5/8 samples, while the catalytic isoforms p110α, p110β and p110δ showed increased expression in 4/8, 6/8 and 1/8 cell lines, respectively (Fig 1C, left panel). In line with the findings in patient samples, cell lines harbouring *MYC* amplification showed decreased levels

of *PIK3CD* mRNA when compared to adrenal tissue (Fig 1C, left panel). As with the patient samples, mRNA levels were largely found to be predictive of protein levels (Fig 1C, right panel).

Insert Figure 1

The expression of PI3K isoforms was next investigated at the protein level in a panel of 8 neuroblastoma cell lines. All neuroblastoma cell lines included in this study expressed the p85α, p110α and p110β isoforms, while the expression of p110δ was more variable, being highest in SH-SY5Y cells (Fig. 2A). Together, these data show that the expression levels of PI3K isoforms are altered in neuroblastoma samples and cell lines at the mRNA and/or protein level, and that the expression of the catalytic p110δ isoform is increased in neuroblastoma.

Insert Figure 2

Activation of PI3K/Akt signalling by polypeptide growth factors in neuroblastoma cell lines. Class I_A PI3Ks transduce signals from activated RTKs to downstream effectors, the most important of which is the Ser/Thr protein kinase Akt (12, 13). The activation of Akt by polypeptide growth factors was investigated in SH-SY5Y and LA-N-1 cells. Epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) most potently activated Akt in SH-SY5Y cells, while platelet-derived growth factor (PDGF) and IGF-1 induced the strongest response in LA-N-1 cells (Fig. 2B).

EGF and IGF-1 stimulated growth of SH-SY5Y (2 and 2.5-fold) and LA-N-1 (2 and 2.2-fold) cells, while PDGF was less potent at promoting a growth response (Fig. 2C). The pharmacological PI3K inhibitor LY294002 reduced basal growth of SH-SY5Y (66.5% inhibition) and LA-N-1 (42.8% inhibition) cells. Treatment with the PI3K inhibitor also reduced EGF- and IGF-1-stimulated growth in SH-SY5Y (44.1% and 27.6% inhibition respectively) and LA-N-1 cells (Fig. 2C). However, LY294002 as a single agent did not significantly affect the extent of growth factor-stimulated cell growth in either SH-SY5Y or LA-N-1 cells (Fig. 2C). Recently, a similar observation was reported in neuroblastoma cell lines, where the combination of both LY294002 and the mTOR inhibitor rapamycin was required to inhibit IGF-1 induced proliferation (9). Thus, various growth factors, including EGF and IGF-1, stimulate growth of neuroblastoma cells via the PI3K/Akt pathway.

Impact of shRNA-mediated down-regulation of p110α and p110δ on neuroblastoma cell proliferation and apoptosis. To gain insight into the individual functions of class I_A PI3K isoforms in neuroblastoma cell responses, SH-SY5Y and LA-N-1 cells were stably transduced with short hairpin RNA (shRNA) constructs targeting p110α (hereinafter $p110\alpha^{low}$) or p110δ (hereinafter $p110\delta^{low}$). Specific down-regulation of target gene expression was verified by Western blot analysis (Fig. 3A).

The growth of SH-SY5Y p110δ^{low} cells was significantly reduced (53.1% and 44.1% inhibition) when the cells were cultivated in medium containing low (1%) serum, but not high (10%) serum (Fig. 3B). This effect was less pronounced in LA-N-1 cells (Fig. 3B), correlating with the differential expression of p110δ in these cell lines. While an effect of p110α down-regulation was also observed in SH-SY5Y cells (low serum: no inhibition or 21.3% inhibition; high serum: no inhibition or 21.2% inhibition), the effect was stronger in LA-N-1 cells (low serum: 22.1% and 66.4% inhibition; high serum: 11.7% and 43.4%,inhibition).

To investigate whether PI3K targeting promoted apoptosis in neuroblastoma cells, caspase activity and cell cycle distribution were analysed in SH-SY5Y p110α^{low} or p110δ^{low} cells. A significant (2.2-fold) increase in caspase-3/7 activity was observed in SH-SY5Y p110δ^{low} cells (Fig. 3C). In contrast, a comparable response was not observed in SH-SY5Y p110α^{low} cells (Fig. 3C). Analysis of cell cycle distribution revealed a decrease in the S-phase in SH-SY5Y p110α^{low} and p110δ^{low} cells, both in low serum (61.8% decrease in both cases) and high serum (71.4% and 59.2% decrease). The increase in cell death under low serum conditions was higher in SH-SY5Y p110δ^{low} cells (319.4% increase) than in SH-SY5Y p110α^{low} cells (64.5% increase), confirming the results of the caspase assay (Fig. 3C). Together, these results demonstrate that the p110δ isoform plays a major role in SH-SY5Y cell growth and survival under suboptimal culture conditions. In contrast, a comparable role was not apparent for p110α in these cells, although this isoform appeared to play a more important function in LA-N-1 cells, which express only very low levels of p110δ.

In view of the increased apoptosis observed in SH-SY5Y p110δ^{low} cells, we analysed the expression of pro- and anti-apoptotic Bcl-2 family proteins, which are key regulators of apoptosis. The levels of Bcl-2 and Bcl-X_L were elevated in SH-SY5Y cells grown in high serum, as compared to low serum conditions (Fig. 3D and 5B). When compared to control cells, SH-SY5Y p110δ^{low} cells displayed reduced expression of the anti-apoptotic proteins Bcl-2 and Bcl-X_L (Fig. 3D and 5B). In contrast, the levels of Bax were unaffected (Fig. 3D). Thus, p110δ appears to contribute to the control of the expression of anti-apoptotic Bcl-2 family proteins, which may impact on its function in neuroblastoma cell survival.

Insert Figure 3

Impact of shRNA-mediated down-regulation of p110α and p110δ on neuroblastoma cell responses to growth factors. We next investigated the involvement of class I_A PI3K isoforms in polypeptide growth factor signalling in neuroblastoma cells. The ability of EGF and IGF-1 to stimulate the growth of SH-SY5Y cells was significantly reduced in both SH-SY5Y p110α^{low} and p110δ^{low} cells (EGF: 6.4% and 40.3%, 23.4% and 16.2%, respectively; IGF-1: 27.5% and 41.5%,

42.1% and 49.1%, respectively) (Fig. 4A). In contrast, the ability of EGF and IGF-1 to stimulate the growth of LA-N-1 p110α^{low} and p110δ^{low} cells was not significantly impaired (Fig. 4A).

The impact of the shRNA constructs targeting p110α and p110δ on the activation of early signalling mediators of RTKs was then investigated in SH-SY5Y and LA-N-1 cells. Activation of Akt by EGF and IGF-1 was unaltered in SH-SY5Y and LA-N-1 cells (Fig. 4B and Supplemental Fig. 2). In contrast, activation of the mTOR/S6K pathway by EGF and IGF-1 was impaired in both SH-SY5Y p110α^{low} and p110δ^{low} cells, as assessed by phosphorylation of the S6 protein (Fig. 4B). While the impact of p110α down-regulation on signal transduction was expected, the observation that decreased p110δ expression leads to a comparable effect on its own was surprising. Activation of Erk1/2 by EGF and IGF-1 was unaffected by the PI3K shRNAs (data not shown). Thus, p110δ plays an important role in activation of the mTOR/S6K pathway in SH-SY5Y cells, which correlates with its involvement in neuroblastoma cell growth. However, activation of Akt by growth factors is insensitive to down-regulation of a single PI3K isoform in these cells. This is not unexpected, as previous studies have reported that PI3K isoforms from other classes contribute to Akt activation (24). In both LA-N-1 p110α^{low} and p110δ^{low} cells, basal activation of Akt and S6K was reduced (Supplemental Fig. 2). However, in LA-N-1 cells, phosphorylation of the S6 protein was hardly affected by down-regulation of p110δ (Supplemental Fig. 2). While decreased p110α expression led to a stronger inhibition of downstream signalling, no apparent effect on the proliferative response was observed, suggesting that other pathways may compensate for reduced signal transduction in this cell line (Fig. 4A and Supplemental Fig. 2).

Insert Figure 4

Constitutive activation of the S6K pathway abrogates the effect of p110δ shRNA on SH-SY5Y cell growth. Our previous data had shown a marked effect of p110δ down-regulation by shRNA on activation of the mTOR/S6K pathway in SH-SY5Y cells, contributing to cell growth. To confirm this model, SH-SY5Y p110δ^{low} cells were transiently transfected with an activated mutant of S6K1. Activated S6K promoted growth of SH-SY5Y cells and was able partially rescue the growth of SH-SY5Y p110δ^{low} cells (Fig. 5A). However, the induction of apoptosis induced by the p110δ shRNA was only marginally impaired by S6K transfection, as assessed by cleavage of poly (ADP-ribose) polymerase (PARP) (Fig. 5B). Moreover, transient transfection of activated S6K did not restore the levels of Bcl-2 and Bcl-X_L in SH-SY5Y p110δ^{low} cells (Fig. 5B). Together, these results demonstrate that the PI3K p110δ isoform controls neuroblastoma cell growth via the S6K pathway. In addition, it also appears to play a role in controlling the levels of Bcl-2 family proteins, which appears not to involve S6K or Akt.

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Insert Figure 5

Rapamycin mimics the effects of p110δ down-regulation. The data presented above highlighted the importance of signalling via the mTOR/S6K pathway in the regulation of neuroblastoma cell survival and the ability to respond to growth factors. To confirm these findings, SH-SY5Y and LA-N-1 cells were treated with rapamycin, an mTOR inhibitor, and cellular responses were investigated. As expected, phosphorylation of the S6 protein was abrogated in the presence of rapamycin, both in low (1%) and high (10%) serum conditions (Fig. 6A). Treatment of SH-SY5Y and LA-N-1 cells with rapamycin led to a dose-dependent inhibition of basal cell proliferation (SH-SY5Y: 29.2% (20ng/ml) and 56.6% (100ng/ml) inhibition; LA-N-1: 37.3% (20ng/ml) and 61.8% (100ng/ml) inhibition) (Fig. 6B). Co-treatment of cells with rapamycin attenuated the proliferative response to EGF (SH-SY5Y: 34.7% (20ng/ml) and 55.8% (100ng/ml) inhibition; LA-N-1: 32.5% (20ng/ml) and 44.4% (100ng/ml) inhibition) and IGF-1 (SH-SY5Y: 32.5% (20ng/ml) and 53% (100ng/ml) inhibition; LA-N-1: 27.7% (20ng/ml) and 42.1% (100ng/ml) inhibition) (Fig. 6B). Furthermore, phosphorylation of the S6 protein was found to be absent upon stimulation with EGF or IGF-1 in cells pre-treated with rapamycin (Fig. 6C). These findings emphasise the importance of intact mTOR/S6K signalling in neuroblastoma cell survival.

Insert Figure 6

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Discussion

In recent years, PI3K has emerged as a central controller of the cellular responses to a variety of growth factors and PI3K signalling is frequently deregulated by diverse mechanisms in human cancer (14, 16). PI3K has also been shown to play an important role in neuroblastoma biology. Therefore, targeting PI3K may represent an attractive approach to inhibit neuroblastoma proliferation *in vivo* (11, 17, 18, 25). However, the mechanisms contributing to the activation of PI3K signalling in neuroblastoma are still unclear, since loss of *PTEN* or *PIK3CA* mutations are uncommon in this paediatric malignancy (26-28). We show here for the first time that the class I_A PI3K isoform p110δ plays a crucial role in neuroblastoma cell growth and survival and that the expression of p110δ is increased at the mRNA and protein level in a subset of primary neuroblastoma samples and cell lines. In addition, our data reveal a selective expression of p85α/p110δ in neuroblastoma samples from children under the age of one. Our results also reveal a negative correlation between *PIK3CD* gene expression and *MYCN* amplification. Expression of *PIK3CD* was recently shown to be significantly lower in neuroblastoma samples with LOH at 1p36 (29). A recent study also described a correlation between decreased *PIK3CD* expression, 1p deletion and poor clinical outcome (30). LOH at 1p36 was associated with amplification of the *MYCN* oncogene (31). Reduced expression of *PIK3CD* in neuroblastoma samples may thus correlate with poor prognosis, due to its association with age at diagnosis (>1 year), LOH at 1p36 and/or *MYCN* amplification, since these events are prognostic markers of poor outcome in neuroblastoma (1, 2, 31, 32). N-Myc was shown to be able to replace IGF/PI3K signalling in medulloblastoma formation (33). Moreover, c-Myc expression had a negative impact on the activation of PI3K/Akt signalling (34). Thus, it is conceivable that *MYCN*-amplified neuroblastoma display decreased *PIK3CD* expression, either because of: (i) LOH at 1p36 (29); (ii) a direct negative effect of *MYCN* amplification on *PIK3CD* expression; or (iii) because they are less dependent on p85α/p110δ signalling. Collectively, these data indicate that the class I_A p110δ isoform may play a role in the development of neuroblastoma in very young patients (<1 year of age), or in a subset of tumours which do not harbour LOH at 1p36 and/or *MYCN* amplification.

The expression of p110δ was previously shown to be predominantly restricted to leukocytes in normal tissues (35). However, different human cancer cell lines were also shown to express this PI3K isoform, indicating that aberrant expression of p110δ in tumours may contribute to the malignant properties of the cancer cells (24, 36). In support of this notion, the ability of p110δ to induce transformation of chicken fibroblasts was recently demonstrated (37). In breast cancer cell lines, a selective function of p110δ in cell migration was also described (36). In acute myeloid leukemia, recent reports have shown that p110δ plays a major role in activation of Akt by RTKs (FLT3), cell proliferation and chemoresistance (38, 39). We show here that the class I_A PI3K isoforms p110α and p110δ do not have overlapping functions in neuroblastoma cell responses. Indeed, in SH-

SY5Y cells which display increased *PIK3CA* and *PIK3CD* expression, p110δ appeared to play a major role in controlling neuroblastoma cell growth and survival under limiting growth conditions. In contrast, in LA-N-1 cells with only very low p110δ expression, down-regulation of p110α by shRNA impaired cell growth and Akt activation. The impact of p110δ down-regulation by shRNA on SH-SY5Y growth correlated with an impairment of the activation of the mTOR/S6K pathway. Surprisingly, Akt activation by EGF and IGF-1 was unaffected by p110δ down-regulation, under conditions where the mTOR/S6K pathway was inhibited. Thus, activation of Akt and mTOR/S6K have different sensitivities to class I_A PI3K down-regulation. It is conceivable that other PI3K isoforms may compensate for p110α or p110δ in growth factor-stimulated Akt activation (24). Transfection of an activated mutant of S6K was sufficient to partially rescue the growth defect of SH-SY5Y $p110\delta^{low}$ cells under low serum conditions. However, p110δ appeared to have an additional function in maintaining the levels of anti-apoptotic Bcl-2 family proteins in neuroblastoma cells, which did not involve S6K or Akt. The observation that the expression levels of anti-apoptotic Bcl-2 family proteins were higher in SH-SY5Y under high serum conditions, than in low serum, indicates that growth factors contribute to the regulation of the levels of Bcl-2 family proteins in these cells. Previous reports have documented a role for IGF-1, which is present in the FCS, in maintaining Bcl-2 (40) and Bcl-X_L (41) expression levels. In view of the data presented here showing a role for p110δ in IGF-1 signalling in neuroblastoma cells, it can be postulated that IGF-1R/p110δ contributes to regulation of the expression of Bcl-2 family proteins. Since Akt and S6K were not involved in this pathway, the simplest explanation for these observations is that mTOR-mediated phosphorylation of 4E-BP1 controls translational activation of the expression of Bcl-2 and/or Bcl-X_L in neuroblastoma cells, which is supported by previous findings in other systems (42). Down-regulation of p110α by shRNA had a less pronounced impact on the growth of SH-SY5Y cells under low serum conditions, although activation of S6K was impaired. In view of the observation that p110α down-regulation did not induce apoptosis in SH-SY5Y cells, in contrast to p110δ, it can be speculated that Bcl-2 family proteins are selectively controlled by the p110δ isoform.

Together, our results demonstrate that p110δ contributes to neuroblastoma cell growth and survival by regulating the activation of the mTOR/S6K pathway and the expression levels of anti-apoptotic Bcl-2 family proteins.

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Figure Legends

Fig. 1. Expression of class I_A PI3K genes in neuroblastoma patient samples and cell lines. *A,* TaqMan analysis of class I_A PI3K mRNA levels in neuroblastoma patient samples (n=19) and human adrenal gland (AG). *PIK3R1*: black bars, *PIK3CA*: light grey bars, *PIK3CB*: white bars, *PIK3CD*: dark grey bars (*left panel*). Western blot of p110δ expression in patient samples (n=12) and AG compared to TaqMan data (*right panel*) *B,* Box plot visualising p85α and p110δ expression in an additional, independent group of 68 human neuroblastoma samples. *D*, Analysis of class I_A PI3K mRNA levels in human neuroblastoma cell lines (n=8) and AG Colour code according to *A* (*left panel*). Western blot of p110δ expression in cell lines (n=8) and AG compared to TaqMan data (*right panel*).

Fig. 2. PI3K class I_A isoform expression and growth factor-induced pathway activation in human neuroblastoma cell lines. *A,* Expression pattern of PI3K isoforms, Akt and the PI3K antagonist phosphatase and tensin homolog (PTEN) in human neuroblastoma cell lines. *B,* Growth factor-induced pathway activation in SH-SY5Y and LA-N-1 cells. *C,* Effect of LY294002 on growth factor-stimulated proliferation.

Fig. 3. Effect of class I_A PI3K isoform down-regulation on cell proliferation and cell death in human neuroblastoma cells. *A,* Western blot analysis of protein down-regulation in SH-SY5Y and LA-N-1 cells stably transfected with shRNA against p110α (PIK3CA) or p110δ (PIK3CD) or the control vector (pRS). *B,* Basal proliferation of SH-SY5Y or LA-N-1 cells in medium containing low (1%) or high (10%). *C*, Basal caspase-3/7 activity of SH-SY5Y clones in medium containing low (1%) or high (10%) serum (*left panel*). Cell cycle distribution of SH-SY5Y clones in medium containing low (1%) serum. Dead cells: black fraction; G2-phase: dark grey fraction; S-phase: white fraction; G0/G1-phase: light grey fraction (*right panel*) *D*, Western blot analysis of the expression levels of proteins involved in apoptosis in SH-SY5Y cells stably transfected with shRNA against p110δ (PIK3CD) or the control vector (pRS) under low (1%) and high (10%) serum conditions.

Fig. 4. Proliferative response and pathway activation upon growth factor stimulation in cells expressing decreased levels of p110α or p110δ. *A*, Growth factor-induced proliferation of SH-SY5Y and LA-N-1 cells stably transfected with shRNA against p110α (light grey bars) or p110δ (dark grey bars) or the control vector pRS (black bars). *B*, Growth factor-induced activation of the PI3K pathway in SH-SY5Y $p110\alpha^{low}$ (PIK3CA) or $p110\delta^{low}$ (PIK3CD) cells compared to control cells (pRS).

Fig. 5. Constitutively activated S6 kinase can partially rescue SH-SY5Y cells from cell death induced by p110δ down-regulation. *A*, Proliferative response of SH-SY5Y $p110\delta^{low}$ cells (PIK3CD) transiently

transfected with constitutively activated S6 kinase 1 (S6K AK1) or the control vector (pcDNA3). *B,* Western blot analysis of the expression levels of proteins involved in apoptosis (Bcl2, Bcl-X_L, Bax) and PARP cleavage.

Fig. 6. Rapamycin mimics the effect of p110δ down-regulation. *A,* Western blot analysis of S6 protein phosphorylation upon treatment of SH-SY5Y and LA-N-1 cells with rapamycin. *B*, Proliferation of SH-SY5Y and LA-N-1 cells in response to EGF and IGF-1 upon co-treatment with rapamycin (ng/ml where indicated). *C*, Western blot analysis of S6 protein phosphorylation in response to stimulation with EGF or IGF-1 upon pre-treatment with rapamycin.

Supplemental Fig. 1. Characteristics of human neuroblastoma patient samples as determined by histological assessment of the tumor specimen obtained at surgery. nd: not determined

Supplemental Fig. 2. Growth factor-induced activation of the PI3K pathway in LA-N-1 p110α^{low} (PIK3CA) or p110δ^{low} (PIK3CD) cells compared to control cells (pRS).

Figure 1

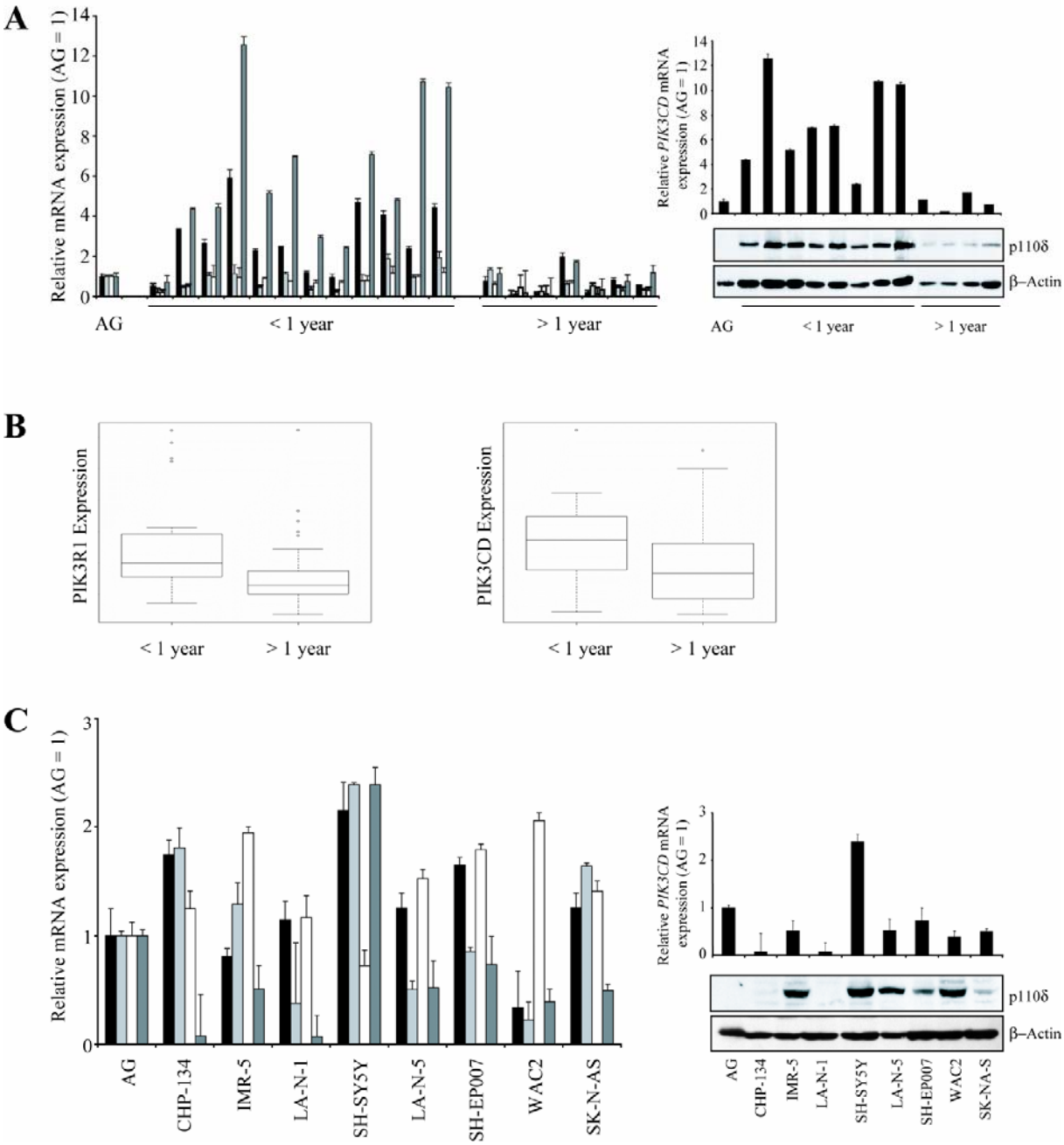

A
Relative mRNA expression (AG = 1)
AG
< 1 year
> 1 year
Relative PIK3CD mRNA expression (AG = 1)
p110δ
β–Actin
B
PIK3R1 Expression
PIK3CD Expression
C
Relative mRNA expression (AG = 1)
AG
CHP-134
IMR-5
LA-N-1
SH-SY5Y
LA-N-5
SH-EP007
WAC2
SK-N-AS
SK-NA-S

Figure 2

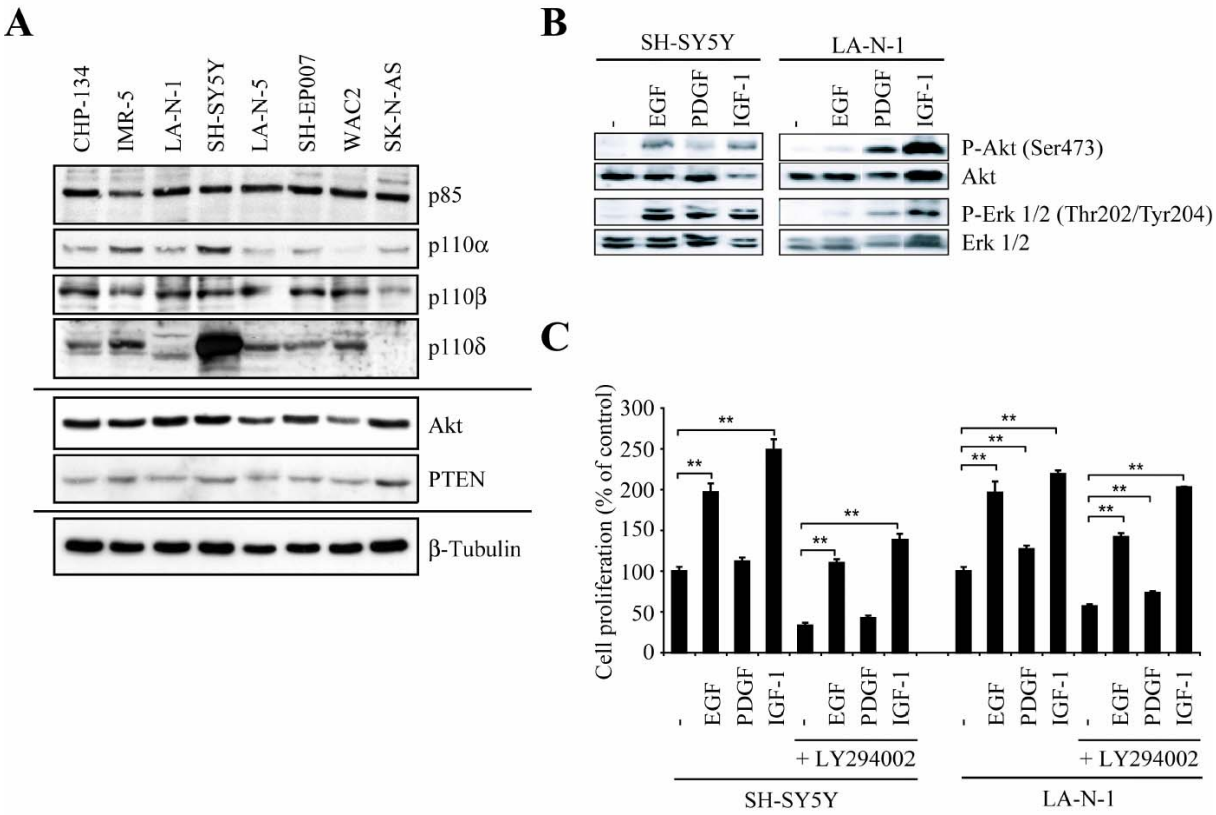

A
CHP-134
IMR-5
LA-N-1
SH-SY5Y
LA-N-5
SH-EP007
WAC2
SK-N-AS
p85
p110α
p110β
p110δ
Akt
PTEN
β-Tubulin
B
SH-SY5Y
LA-N-1
-
EGF
PDGF
IGF-1
P-Akt (Ser473)
Akt
P-Erk 1/2 (Thr202/Tyr204)
Erk 1/2
C
Cell proliferation (% of control)
300
250
200
150
100
50
0
**
+ LY294002
SH-SY5Y
LA-N-1

Figure 3

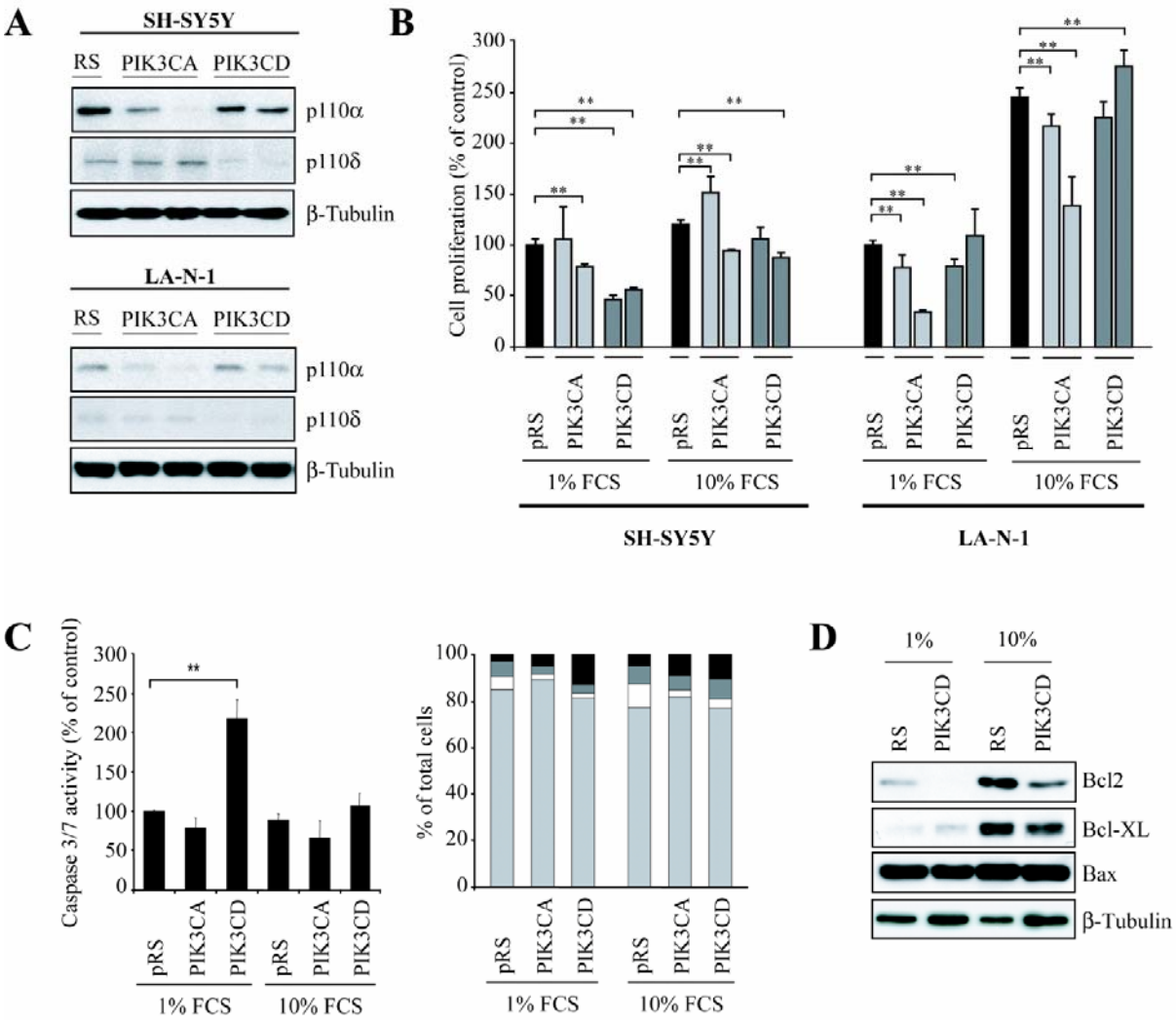
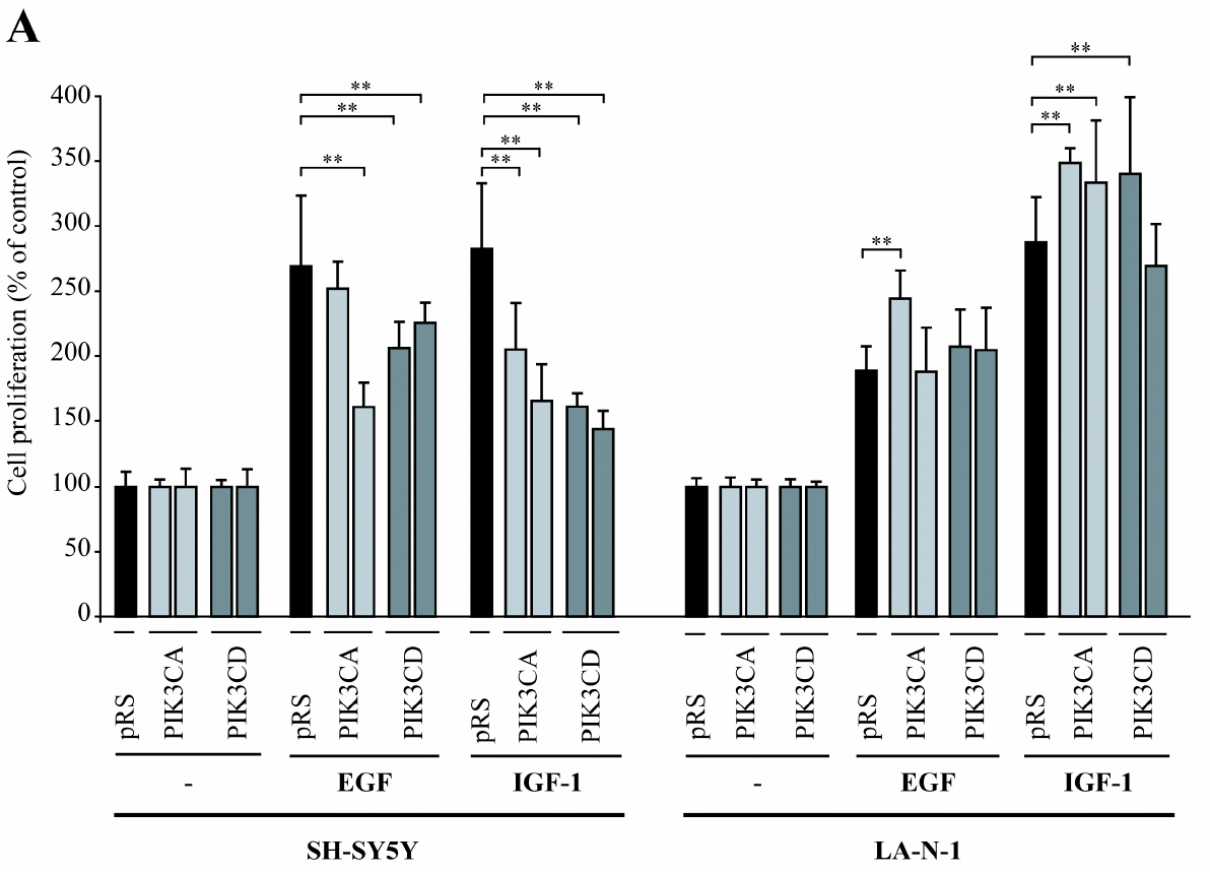

A
SH-SY5Y
RS
PIK3CA
PIK3CD
p110α
p110δ
β-Tubulin
LA-N-1
RS
PIK3CA
PIK3CD
p110α
p110δ
β-Tubulin
B
Cell proliferation (% of control)
300
250
200
150
100
50
0
pRS
PIK3CA
PIK3CD
1% FCS
10% FCS
SH-SY5Y
LA-N-1
C
Caspase 3/7 activity (% of control)
% of total cells
D
1%
10%
RS
PIK3CD
Bcl2
Bcl-XL
Bax
β-Tubulin

Figure 4


A
Cell proliferation (% of control)
400
350
300
250
200
150
100
50
0
**
pRS
PIK3CA
PIK3CD
-
EGF
IGF-1
SH-SY5Y
LA-N-1

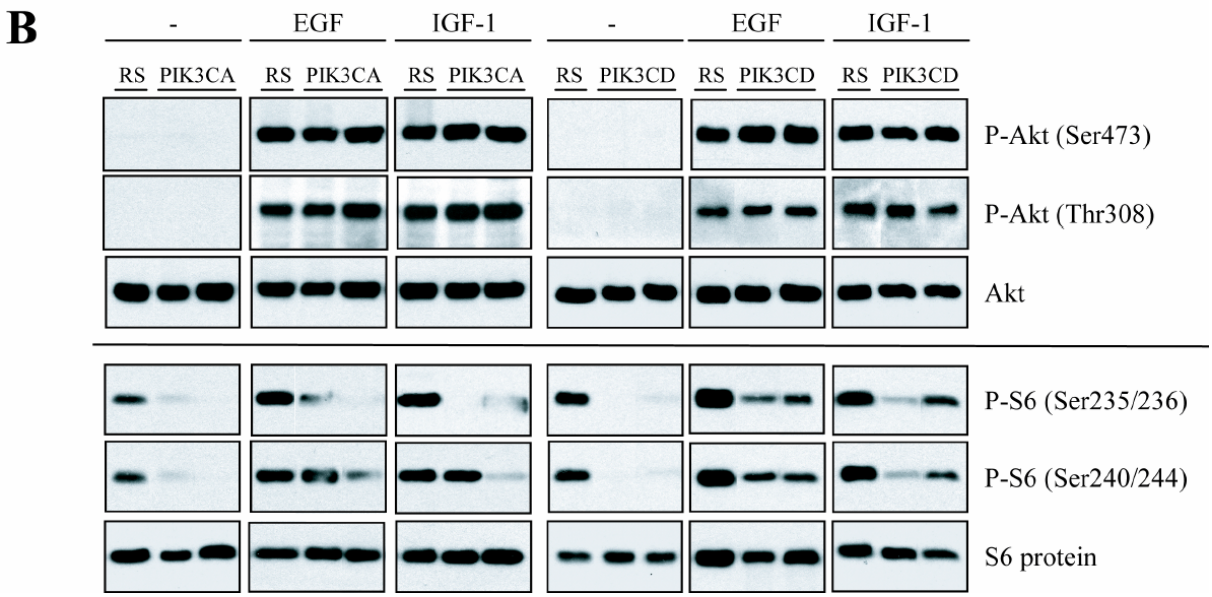

B
-
EGF
IGF-1
RS
PIK3CA
PIK3CD
P-Akt (Ser473)
P-Akt (Thr308)
Akt
P-S6 (Ser235/236)
P-S6 (Ser240/244)
S6 protein

Figure 5

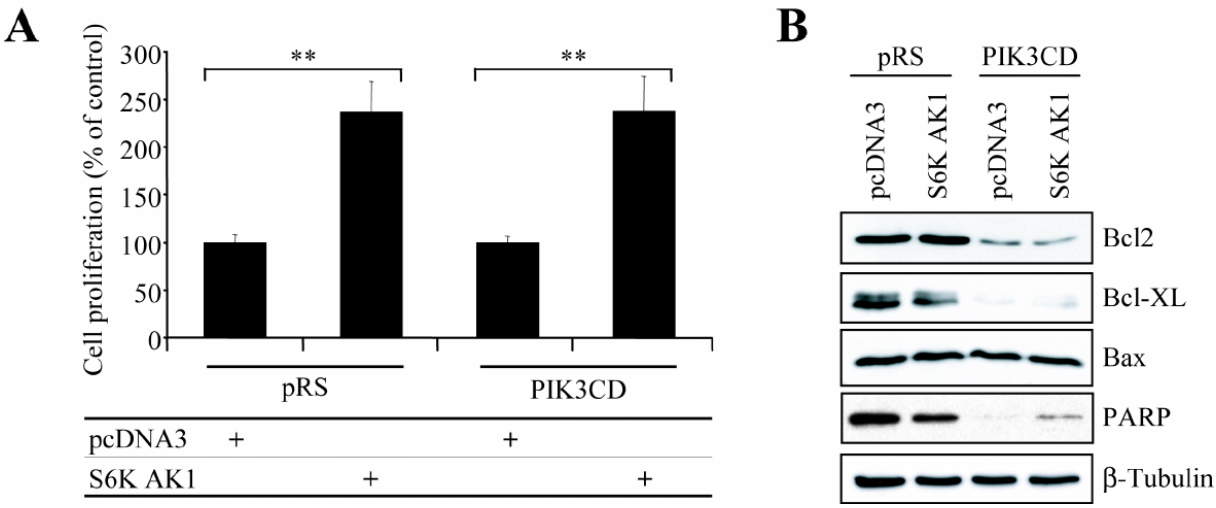
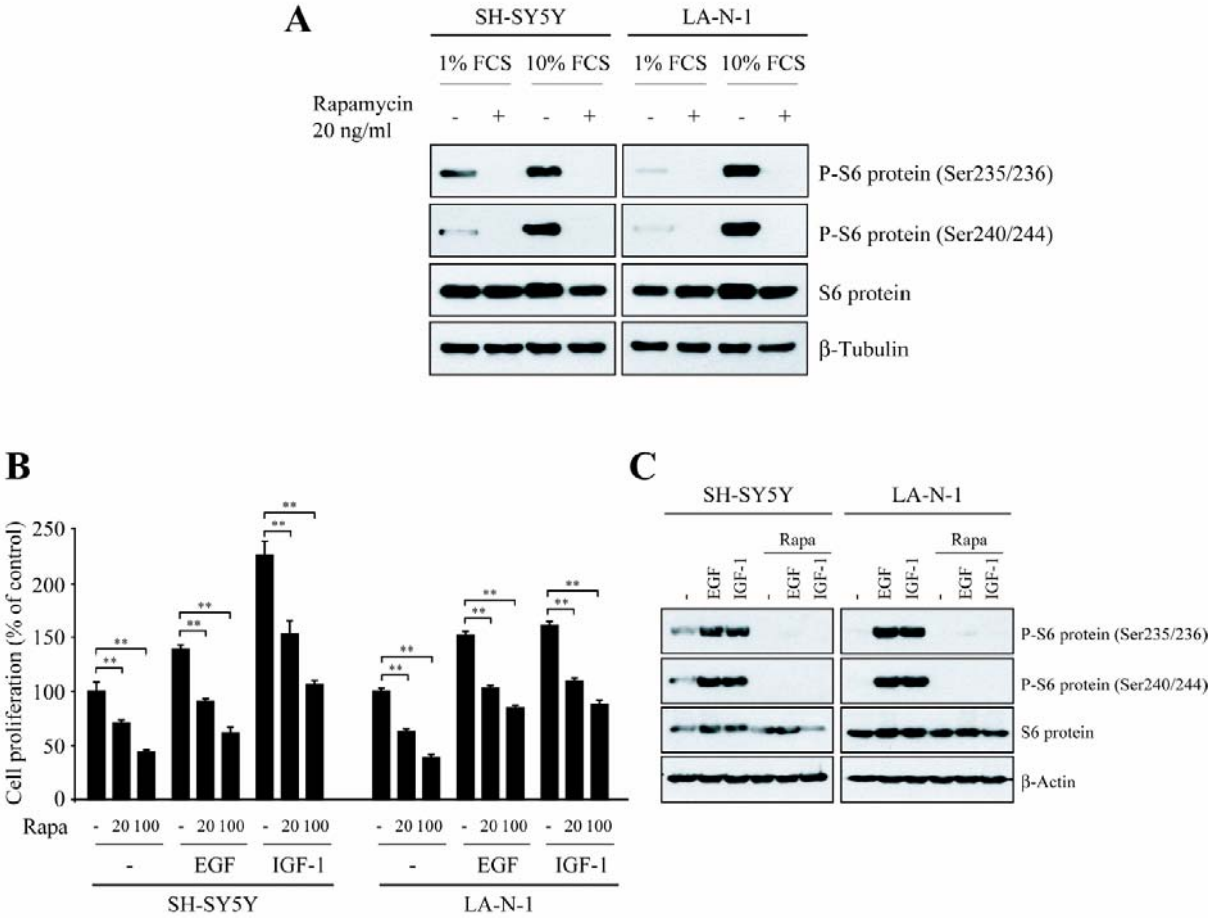

A
Cell proliferation (% of control)
300
250
200
150
100
50
0
**
**
pRS
PIK3CD
pcDNA3 + +
S6K AK1 + +
B
pRS
PIK3CD
pcDNA3
S6K AK1
pcDNA3
S6K AK1
Bcl2
Bcl-XL
Bax
PARP
β-Tubulin

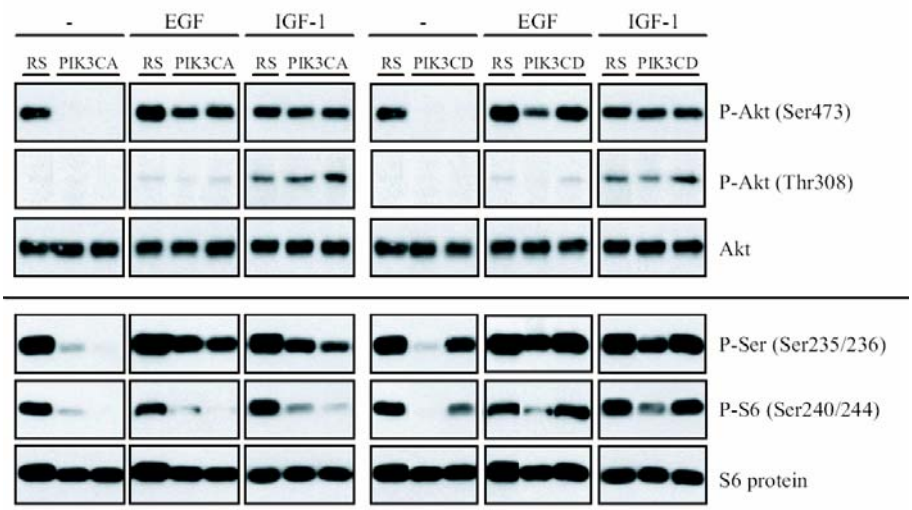
Figure 6


A
SH-SY5Y
LA-N-1
1% FCS
10% FCS
1% FCS
10% FCS
Rapamycin 20 ng/ml
- + - + - + - +
P-S6 protein (Ser235/236)
P-S6 protein (Ser240/244)
S6 protein
β-Tubulin
B
Cell proliferation (% of control)
250
200
150
100
50
0
Rapa
- 20 100 - 20 100 - 20 100 - 20 100 - 20 100 - 20 100
-
EGF
IGF-1
-
EGF
IGF-1
SH-SY5Y
LA-N-1
C
SH-SY5Y
LA-N-1
Rapa
Rapa
- EGF IGF-1 - EGF IGF-1 - EGF IGF-1 - EGF IGF-1
P-S6 protein (Ser235/236)
P-S6 protein (Ser240/244)
S6 protein
β-Actin

Supplemental Figure 1

Sample Nr.	Age at diagnosis (years)	Stage	MYC status 0 - single copy 1 - amplified	1p status 0 - normal 1 - deleted	Progression 0 - no 1 - yes
1	0.0	4S	0	nd.	0
2	1.0	2	0	1	0
3	0.2	4S	0	0	0
4	0.1	3	0	0	0
5	0.2	3	0	0	0
6	0.5	2	0	0	0
7	0.7	3	1	0	0
8	0.1	4	1	1	1
9	0.4	4	0	0	0
10	0.4	3	0	0	1
11	0.4	2	0	0	0
12	0.0	1	0	1	0
13	3.1	4	0	0	1
14	4.0	2	1	1	0
15	1.3	3	1	1	0
16	5.1	4	0	1	1
17	2.2	2	0	0	0
18	2.8	2	1	0	1
19	2.4	4	0	0	0

Supplemental Figure 2



3.2.2 Protein kinase B modulates the sensitivity of human neuroblastoma cells to insulin-like growth factor inhibition

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Protein kinase B modulates the sensitivity of human neuroblastoma cells to insulin-like growth factor receptor inhibition

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The potential of the novel insulin-like growth factor receptor (IGF-IR) inhibitor NVP-AEW541 as an antiproliferative agent in human neuroblastoma was investigated. Proliferation of a panel of neuroblastoma cell lines was inhibited by NVP-AEW541 with IC_{50} values ranging from 0.15 to 5 μM. Experiments using an IGF-IR neutralizing antibody confirmed that the IGF-IR was essential to support growth of neuroblastoma cell lines. The expression levels of the IGF-IR in individual neuroblastoma cell lines did not correlate with the sensitivities to NVP-AEW541, while coexpression of the IGF-IR and the insulin receptor (IR) correlated with lower sensitivity to the inhibitor in some cell lines. Intriguingly, high levels of activation of Akt/protein kinase B (PKB) and phosphorylation of the ribosomal S6 protein were observed in neuroblastoma cell lines with decreased sensitivities to NVP-AEW541. Inhibition of Akt/PKB activity restored the sensitivity of neuroblastoma cells to the IGF-IR inhibitor. Transfection of neuroblastoma cells with activated Akt or ribosomal protein S6 kinase (S6K) decreased the sensitivity of the cells to NVP-AEW541. IGF-I-stimulated proliferation of neuroblastoma cell lines was completely blocked by NVP-AEW541, or by a combination of an inhibitor of phosphoinositide 3-kinase and rapamycin. In addition to its antiproliferative effects, NVP-AEW541 sensitized neuroblastoma cells to cisplatin-induced apoptosis. Together, our data demonstrate that NVP-AEW541 in combination with Akt/PKB inhibitors or chemotherapeutic agents may represent a novel approach to target human neuroblastoma cell proliferation.

Key words: insulin-like growth factor; neuroblastoma; Akt/protein kinase B

Neuroblastoma is the most common extra cranial solid tumor occurring in children and accounts for about 10% of pediatric malignancies.[1,2] Current treatments of neuroblastoma such as radiotherapy and chemotherapy are inefficient in subgroups of tumors, because of the resistance of the tumor cells to apoptotic signals. Treatment of high-risk neuroblastoma with prolonged intensive chemotherapy and radiotherapy including peripheral stem cell rescue resulted only in 34% 3-year event-free survival probability.[3]

Promising new therapies for neuroblastoma are, however, emerging, which are based on blocking receptor tyrosine kinase (RTK) signaling to some of their downstream signaling targets such as phosphoinositide 3-kinase (PI3K), protein kinase B (PKB)/Akt, the mammalian target of rapamycin (mTOR), the ribosomal protein S6 kinase (S6K) or mitogen-activated extracellular signal-regulated kinase activating kinase (MEK). Polypeptide growth factors have indeed been shown to play a key role in neuroblastoma proliferation, chemoresistance and metastasis. IGF signaling has been extensively studied in the context of neuroblastoma proliferation, survival and motility.[4–7] Several potential anti-neuroblastoma therapeutic approaches involving the IGF-I system have been reported.[8] Moreover, inhibition of platelet-derived growth factor receptor (PDGFR) and c-Kit signaling with imatinib mesylate was recently reported to impair growth of a subset of neuroblastoma cell lines.[9] Neurotrophins such as brain-derived neurotrophic factor (BDNF) also play an important role in neuroblastoma chemoresistance by binding to the Trk receptor family.[10] Fibroblast growth factor-2 (FGF-2), which is expressed in human neuroblastoma cells, may also have chemoprotective and radioprotective effects, given its antiapoptotic functions in other human cancers.[11–13] Finally, an autocrine loop between stem cell factor (SCF) and c-Kit is present in neuroblastoma.[14] Therefore, given that neuroblastoma express a variety of different growth factor receptors, targeting individual receptors may not necessarily provide a successful therapeutic strategy. An alternative approach would be to identify a signaling molecule which lies downstream of several different growth factor receptors and which is essential for transmitting their proliferative and survival message.

PI3K is a good example of this, playing a crucial role in controlling cell proliferation, survival and motility/metastasis downstream of many different growth factor receptors.[15,16] The importance of PI3K signaling in human cancer is highlighted by the fact that mutations in the tumor suppressor gene *PTEN* occur frequently in human tumors. PTEN is a phosphatase that antagonizes the action of PI3K by dephosphorylating the D-3 position of polyphosphoinositides.[17] Moreover, recent reports have described activating mutations in the *PIK3CA* gene encoding the catalytic p110α isoform of PI3K in a variety of human cancers, including breast, colon and ovarian cancer, as well as medulloblastoma.[18] In neuroblastoma, BDNF was shown to protect the tumor cells from chemotherapy-induced apoptosis via the PI3K pathway.[19] IGF-I signaling via PI3K was shown to be required for neuroblastoma differentiation and cytoskeletal rearrangements.[20] Thus, targeting the PI3K/Akt/mTOR/S6K pathway may also represent an attractive novel approach to develop therapies for neuroblastoma.

In the present report we have evaluated the potential of the novel IGF-IR inhibitor NVP-AEW541[21] as an antitumor agent in neuroblastoma. Moreover, we have investigated whether targeting downstream signaling mediators of the IGF-IR could enhance the efficacy of the inhibitor in resistant neuroblastoma cell lines.

Materials and methods

Reagents and antibodies

Antibodies against caspase-3, Erk1/2, IGF-IRα and β, IRβ, PARP, Akt/PKB were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against activated Erk1/2 (Thr202/Tyr204), phosphorylated IGF-IR (Tyr1131), activated Akt/PKB (Ser473) and Ser235/236- and Ser240/244-phosphorylated S6 protein were from Cell Signalling Technology (Danvers,

Abbreviations: BDNF, brain-derived neurotrophic factor; EGFR, epidermal growth factor receptor; Erk, extracellular signal-regulated kinase; FGF-2, fibroblast growth factor-2; IGF-IR, insulin-like growth factor-I receptor; IR, insulin receptor; IRS-1, insulin receptor substrate-1; MEK, mitogen-activated Erk kinase; mTOR, mammalian target of rapamycin; PARP, poly(ADP-ribose) polymerase; PDGFR, platelet-derived growth factor receptor; PDK1, phosphoinositide-dependent kinase-1; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; RTK, receptor tyrosine kinase; S6K, ribosomal protein S6 kinase; SCF, stem cell factor; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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E-mail: alexandre.arcaro@kispi.unizh.ch

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MA, USA). The Akt inhibitor (1L-6-hydroxymethyl-*chiro*-inositol 2-(R)-2-*O*-methyl-3-*O*-octadecylcarbonate), IGF-I, the IGF-IR neutralizing antibody, LY294002, rapamycin and PD98059 were from Calbiochem (La Jolla, CA, USA). The anti-actin and anti-β-tubulin I antibodies and insulin were from Sigma Aldrich (St. Louis, MO, USA). NVP-AEW541 (Novartis Pharma AG, Basel, Switzerland) was dissolved in DMSO at 10 mM and diluted into cell culture medium just prior to use.

Cell culture

Human neuroblastoma cell lines were grown in RPMI (Life Technologies/Invitrogen, Carlsbad, CA, USA) with 10% (v/v) FCS (fetal calf serum) and penicillin/streptomycin/L-glutamine and passaged every 3–5 days by trypsinization. For serum-starving, the cells were incubated for 16 hr in RPMI containing 1.0 % (v/v) FCS.

Cell proliferation

Neuroblastoma cell lines (5×10^4 cells/dish) were seeded in 35-mm dishes and grown for 3 days in serum (FCS 10%)-containing medium in the presence or absence of inhibitors. The cells were then harvested by trypsinization, disaggregated by passing through a 22G needle and viable cells counted with a haemocytometer using Trypan blue exclusion.

Alternatively, neuroblastoma cell lines (5×10^3 cells/well) were seeded in 96-well plates and grown for 3 days in serum (10%)-containing medium in the presence or absence of inhibitors. For growth factor stimulations, cells were incubated in medium containing 1% FCS. Cell proliferation was analyzed by MTS assay using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions.

For DNA synthesis measurements, neuroblastoma cell lines (5×10^4 cells/dish) were seeded in 35-mm dishes and grown for 20 hr in serum (FCS 10%)-containing medium in the presence or absence of inhibitors. For growth factor stimulations, cells were incubated in medium containing 1% FCS. [^{3}H] thymidine (Amersham Biosciences, Buckinghamshire, UK) was then added (1 μCi/dish) for 4 hr. The cells were then washed 3 times with 2 ml of cold phosphate-buffered saline, scraped and lysed in 0.5 ml 0.1 % (w/v) SDS, and the dishes rinsed with 0.5 ml 0.1 % SDS. Bovine serum albumin (0.5 mg/ml final concentration) and trichloroacetic acid (10% (w/v) final concentration) were then added and the samples centrifuged at 12,000*g* for 10 min. The pellets were resuspended in 0.5 ml NaOH (0.1 M) and radioactivity was measured by scintillation counting on a Packard Tricarb 1900CA Beta-Counter (Perkin Elmer Life Sciences, Boston, MA, USA).

Apoptosis

For detection of apoptosis, neuroblastoma cells were incubated for 24 hr in the presence or absence of inhibitors. The cells were then lysed and samples analyzed by SDS-PAGE and Western blot with anti-poly(ADP-ribose) polymerase (PARP) or anti-caspase-3 antibodies. To assess induction of apoptosis in the panel of neuroblastoma cell lines, the Cell Death Detection ELISA^{PLUS} (Roche, Rotkreuz, Switzerland) was used according to the manufacturer's instructions. Alternatively, caspase activity was measured using the CaspACE^{TM} Assay System (Promega) or the Caspase-Glo 3/7 Assay System (Promega) according to the manufacturer's instructions.

Transient and stable expression in neuroblastoma cells

Human neuroblastoma cells were transiently transfected with Akt/PKB constructs in pUSE (Upstate, Charlottesville, VA, USA) constructs using Lipofectamine-Plus (Invitrogen) according to the manufacturer's protocol. Cell responses were assessed 72 hr post-transfection.

For stable expression, SH-SY5Y cells were transfected with Akt/PKB constructs in pUSE (Upstate) or an activated S6K1 construct (T412D) in pCDNA3 (Invitrogen),[22] using Lipofectamine (Invitrogen). After 48 hr, the cells were diluted 1:10 in growth medium containing G418 (0.8 mg/ml). A mixed population of resistant cells was expanded and analyzed after selection.

Statistical analysis

Analysis of variance and Bonferroni multiple comparison test or Kruskal-Wallis non-parametric analysis of variance test were used to assess statistical significance of differences between groups. The non-parametric Mann-Whitney test was used to compare the medians of 2 unpaired groups. p values <0.05 were considered as significant.

Results

Characterization of the expression of RTKs and signaling intermediates in a panel of human neuroblastoma cell lines and correlation with sensitivities of the cells to NVP-AEW541

A panel of 9 low-passage human neuroblastoma cell lines was analyzed for expression of the IGF-IRα and β and IRβ by Western blot analysis of cell lysates. High levels of IGF-IRβ expression were found in the SH-SY5Y, CHP134, IMR5, LAN1 and SKNAS cell lines, while the IR was overexpressed in the CHP134 and IMR5 cell lines (Fig. 1*a*). Western blot analysis also revealed expression of the IGF-IRα in all of the neuroblastoma cell lines, and species of different molecular weights were observed in some of the cell lines, which was potentially caused by heterogeneity in glycosylation. The Western blot analysis revealed no significant differences in expression of the downstream signaling intermediates insulin receptor substrate-1 (IRS-1), PTEN, Akt/PKB and

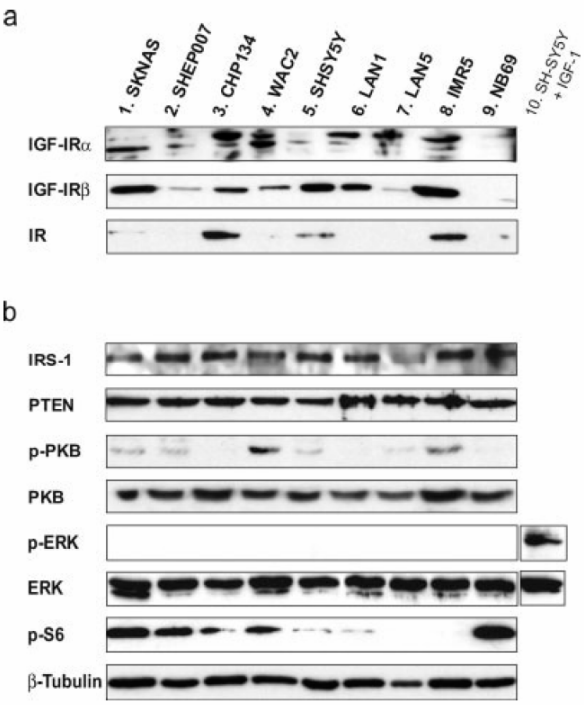

FIGURE 1 – Expression of components of the IGF-I and insulin receptor signaling pathways in human neuroblastoma cell lines. (*a*), (*b*) Equal amounts of lysates from confluent human neuroblastoma cell lines grown in 10% FCS (as indicated) were analyzed by SDS-PAGE and Western blotting with antibodies specific for the proteins indicated. p-PKB: Akt/PKB phosphorylated on Ser473. p-Erk: Erk phosphorylated on Thr202/Tyr204. p-S6: ribosomal S6 protein phosphorylated on Ser240/244. Lysates from IGF-I-stimulated SH-SY5Y cells were analyzed in parallel for the p-Erk blot.

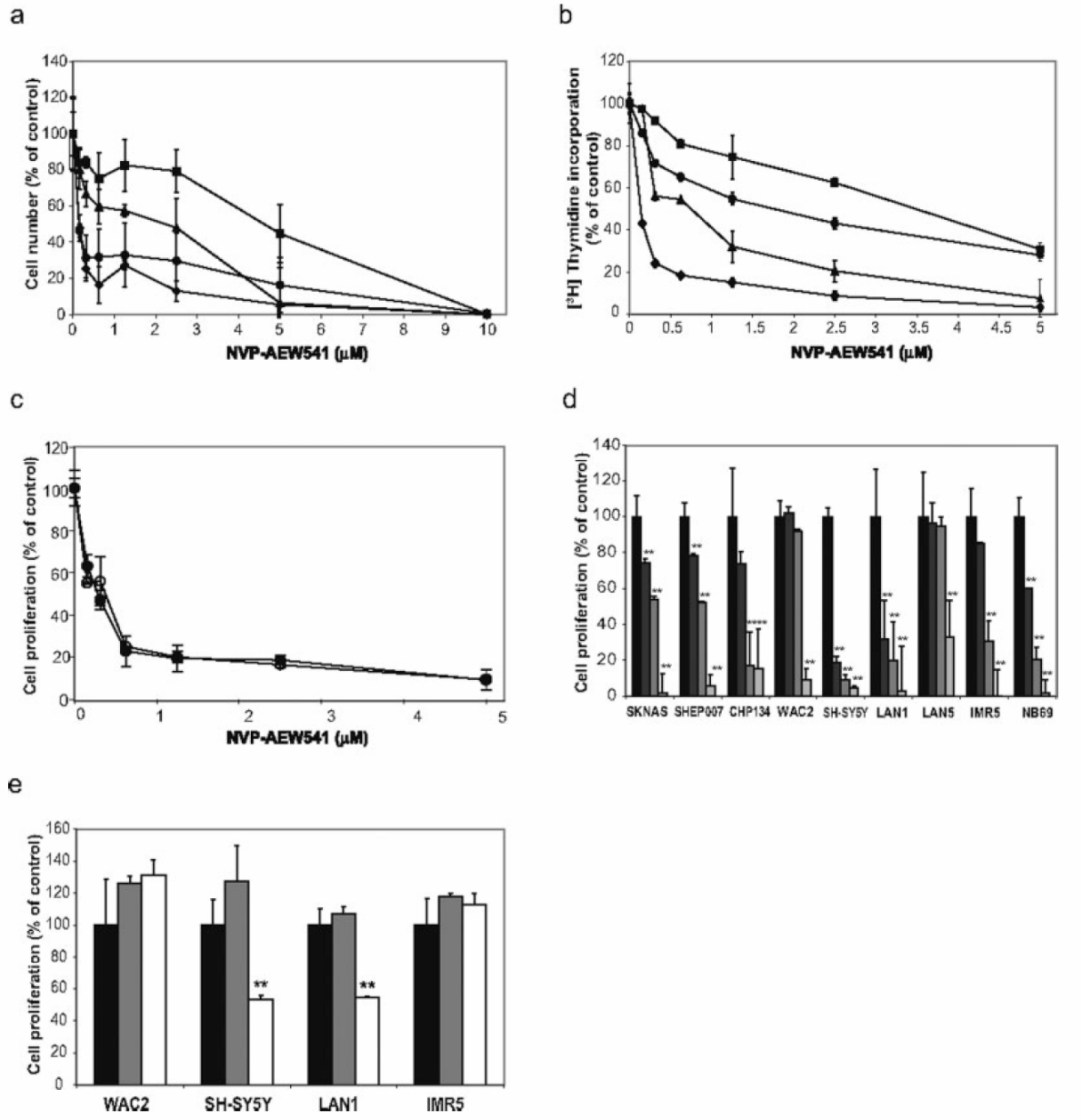

FIGURE 2 – Inhibition of neuroblastoma cell proliferation by an IGF-IR kinase inhibitor or an anti-IGF-IR neutralizing antibody. (*a*) SH-SY5Y (diamonds), LAN1 (circles), CHP134 (triangles) or WAC2 (squares) cells were incubated with increasing concentrations of NVP-AEW541 in medium containing 10% FCS. Cell numbers were determined by counting after 72 hr and expressed as percentage of the control. Data are mean with SD from triplicate determinations. (*b*) Neuroblastoma cells as in (*a*) were incubated with increasing concentrations of NVP-AEW541 and DNA synthesis measured after 24 hr. Data were expressed as percentage of control and are mean with SD from triplicate determinations. (*c*) SH-SY5Y cells were incubated with increasing concentrations of NVP-AEW541 in medium containing 1% (open circles) or 10% FCS (closed circles) and cell proliferation was determined after 72 hr using an MTS assay. Data are mean with SD from 8 repetitions and are expressed as percentage of the control. (*d*) Nine different neuroblastoma cell lines were incubated with vehicle (black bars) or NVP-AEW541 (2.5 μM, dark grey bars; 5 μM, light grey bars; 10 μM, open bars). Cell proliferation was measured as in (*c*) and expressed as percentage of the control. (*e*) WAC2, SH-SY5Y, LAN1 or IMR5 cells were incubated without (black bars) or with anti-IGF-IR neutralizing antibody (1 μg/ml, open bars), or control OKT3 antibody (1 μg/ml, grey bars). Cell proliferation was measured as in (*c*). Data are mean with SD from 8 repetitions. All the experiments were performed at least twice with similar results and 1 representative experiment is shown. ** indicates a significant difference between the control and NVP-AEW541-treated samples, or between the anti-IGF-IR and OKT3-treated samples (**$p < 0.01$).

extracellular signal-regulated kinase (Erk) (Fig. 1*b*). High activation of Akt/PKB was detected in WAC2 and IMR5, while none of the cell lines displayed any detectable activation of Erk1/2 (Fig. 1*b*). High levels of phosphorylation of the S6 protein were also detected in SKNAS, SHEP007, CHP134, WAC2 and NB69 cells (Fig. 1*b*). The WAC2 cell line displayed both Akt activation

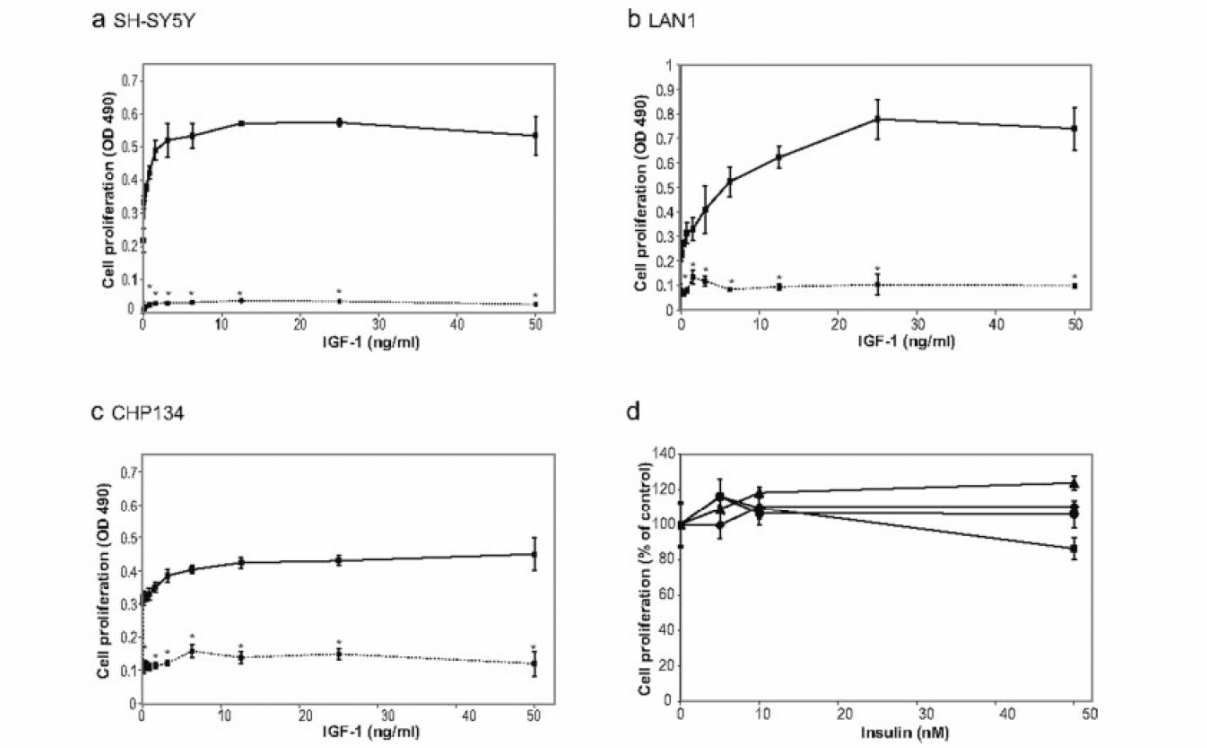

FIGURE 3 – NVP-AEW541 inhibits IGF-I-stimulated cell proliferation in human neuroblastoma cell lines. (*a*)–(*c*) SH-SY5Y (*a*), LAN1 (*b*) or CHP134 (*c*) cells were incubated with increasing concentrations of IGF-I in the presence of vehicle (solid line) or NVP-AEW541 (5 μM, broken line). Cell proliferation was determined after 72 hr using an MTS assay. (*d*) SH-SY5Y (diamonds), LAN1 (circles), CHP134 (triangles) or WAC2 (squares) were incubated with increasing concentrations of insulin. Cell proliferation was measured as in (*a*)–(*c*). Data are mean with SD from 8 repetitions. All the experiments were performed at least twice with similar results and 1 representative experiment is shown. ∗ indicates a significant difference between the control and NVP-AEW541-treated samples ($p < 0.05$).

and S6 protein phosphorylation, while in the other neuroblastoma cell lines activation of both pathways did not match.

The ability of the specific IGF-IR inhibitor NVP-AEW541[21] to block neuroblastoma cell growth in serum-containing medium was then investigated. NVP-AEW541 potently suppressed growth of SH-SY5Y (IC_{50} = 0.15 μM) and LAN1 cells (IC_{50} = 0.15 μM) after 72 hr, but was less efficient at inhibiting growth of CHP134 (IC_{50} = 2.8 μM) and WAC2 (IC_{50} = 5.0 μM) (Fig. 2*a*). These differences in sensitivities to NVP-AEW541 were also observed when DNA synthesis measurements (24-hr assay) were performed in SH-SY5Y and WAC2 cells, demonstrating that the IGF-IR inhibitor impairs proliferation of the cells (Fig. 2*b*). However, in LAN1 cells, the inhibitor affected DNA synthesis at higher concentrations (IC_{50} = 2.0 μM) than in the cell counting assays, while in CHP134 cells it was more effective (IC_{50} = 0.8 μM). These discrepancies may possibly be accounted for by differences in cell death induced by NVP-AEW541, which could make a more prominent contribution to the reduction in cell numbers observed after 72 hr in some of the cell lines (Fig. 6*b*). The sensitivity of neuroblastoma cells toward NVP-AEW541 was not significantly altered when proliferation was measured in medium containing low (1%) serum, as compared to that measured in normal growth medium (10% serum) (Fig. 2*c*). The inhibitor was then tested in a proliferation assay (MTS) on the panel of 9 neuroblastoma cell lines at 3 different concentrations. Varying sensitivities to NVP-AEW541 were found amongst the cell lines, with SH-SY5Y and LAN1 being most sensitive (>50% growth inhibition at 2.5 μM), CHP134, SKNAS (IC_{50} = 2.8 μM), SHEP007 (IC_{50} = 3.6 μM), IMR5 (IC_{50} = 4.6 μM) and NB69 (IC_{50} = 3.6 μM) displaying intermediate sensitivities (>50% growth inhibition at 5.0 μM) and WAC2 and LAN5 (IC_{50} = 5.2 μM) being more resistant (>50% growth inhibition at 10.0 μM) (Fig. 2*d*). Determination of the IC_{50}s for NVP-AEW541 in all the cell lines was done separately by dose-dependency studies using MTS assays (data not shown). The results obtained in the MTS assay were comparable to those of the cell counting assay for CHP134, WAC2, SH-SY5Y and LAN1 in terms of sensitivities to NVP-AEW541 (Figs. 2*a* and 2*d*). Thus the IGF-IR inhibitor NVP-AEW541 inhibits growth and proliferation of human neuroblastoma cell lines with varying efficiencies.

The expression levels of the IGF-IRβ, which is the target of NVP-AEW541, did not correlate with the sensitivities of the neuroblastoma cell lines to the inhibitor (Figs. 1*a* and 2*d*). However, low expression levels of the IGF-IRβ were found in SHEP007, LAN5 and NB69 cells, which displayed reduced sensitivity to the inhibitor (Figs. 1*a* and 2*d*). The highest levels of IR expression were found in some of the cell lines with reduced sensitivity to the inhibitor, such as CHP134 and IMR5 (Figs. 1*a* and 2*d*). Activation of Akt/PKB was also highest in the WAC2 cell line, which was resistant to NVP-AEW541 (Figs. 1*b* and 2*a*), indicating that activation of Akt may render some neuroblastoma cell lines less sensitive to the IGF-IR inhibitor. The enhanced activation of Akt/PKB was observed in WAC2 cells grown in the presence of 10% serum (Fig. 1*a*), but was not detectable in serum-starved cells (Fig. 4*b*).

To confirm the data obtained with the IGF-IR kinase inhibitor, a neutralizing antibody directed against the receptor was tested for its ability to inhibit neuroblastoma cell proliferation. Two NVP-AEW541-sensitive cell lines (SH-SY5Y, LAN1) and 2 cell lines with reduced sensitivity (WAC2, IMR5) were analyzed. A signifi-

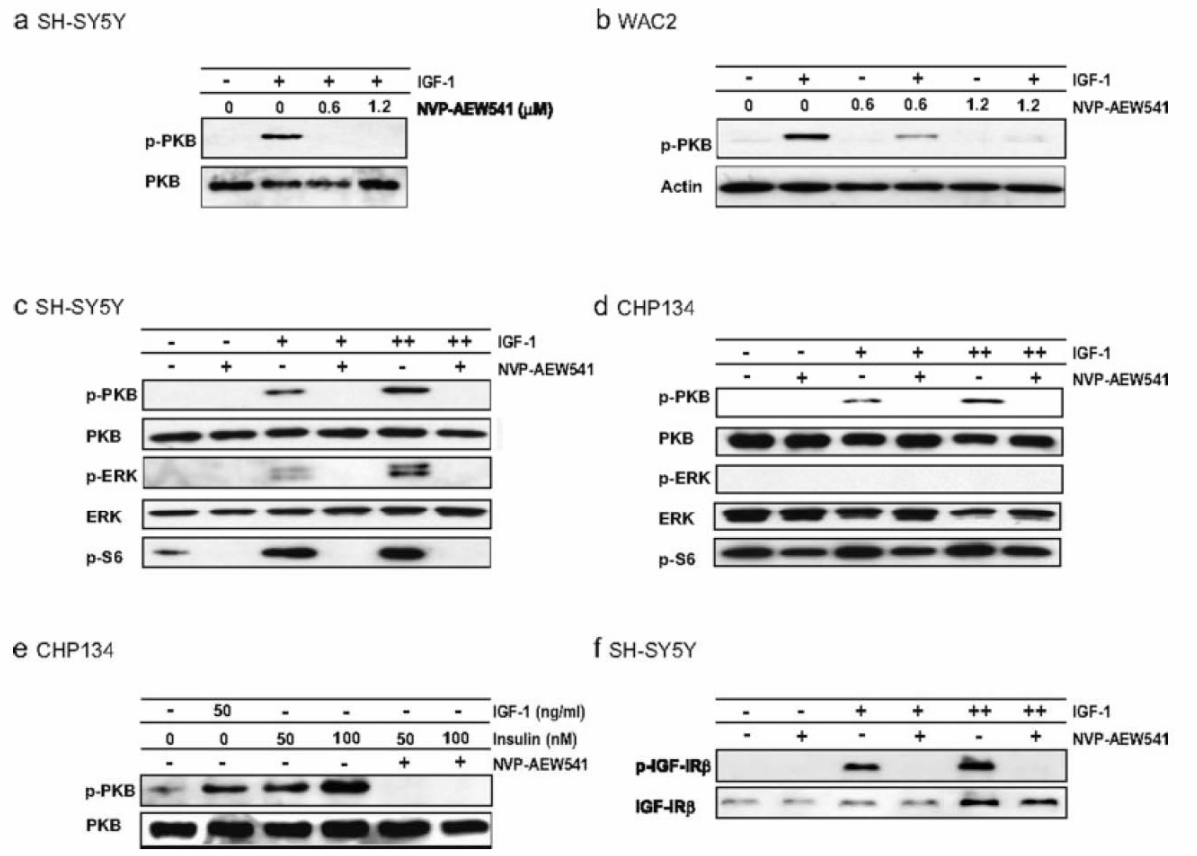

Figure 4 – IGF-I activates Akt/PKB, Erk and S6K in human neuroblastoma cell lines. (*a*), (*b*) Serum-starved SH-SY5Y (*a*) or WAC2 (*b*) cells were pretreated with vehicle or NVP-AEW541 at the concentrations indicated for 30 min and stimulated with IGF-I (50 ng/ml) for 10 min at 37°C. Cell lysates were analyzed by SDS-PAGE and Western blot for the proteins indicated. (*c*)–(*d*) Serum-starved SH-SY5Y (*c*) or CHP134 (*d*) cells were pretreated with vehicle or NVP-AEW541 (2.5 μM) for 30 min and stimulated with IGF-I (+: 25 ng/ml, ++: 50 ng/ml) for 10 min at 37°C. Cell lysates were analyzed by SDS-PAGE and Western blot for the proteins indicated. (*e*) Serum-starved CHP134 cells were pretreated with vehicle (–) or NVP-AEW541 (+: 5 μM) for 30 min and were stimulated with IGF-I or insulin at the concentrations indicated for 10 min at 37°C. Cell lysates were analyzed by SDS-PAGE and Western blot for the proteins indicated. (*f*) SH-SY5Y were pretreated and stimulated as in (*c*) and samples analyzed by SDS-PAGE and Western blot for phosphorylated IGF-IRβ or total receptor.

cant inhibition of cell proliferation was observed when SH-SY5Y or LAN1 cells were treated with the IGF-IR neutralizing antibody, as compared to a control (OKT3) antibody (Fig. 2*e*). In contrast, no differences in cell proliferation were observed in the WAC2 and IMR5 cell lines upon anti-IGF-IR antibody treatment (Fig. 2*e*), confirming the results obtained with NVP-AEW541 (Figs. 2*a* and 2*d*). The inhibition of cell growth observed at higher concentrations (10 μM) of NVP-AEW541 in WAC2 and IMR5 cells may be explained by inhibition of other targets.[21]

Impact of NVP-AEW541 on IGF-I-stimulated cell growth and activation of downstream signaling pathways

The ability of IGF-I to stimulate neuroblastoma cell growth was then investigated in 3 different cell lines. Two NVP-AEW541-sensitive cell lines (SH-SY5Y, LAN1) and 1 cell line with reduced sensitivity (CHP134) were chosen for analysis. A dose-dependent increase in cell proliferation was observed in response to IGF-I in SH-SY5Y (3-fold maximal increase), LAN1 (4-fold increase) and CHP134 (1.5-fold increase) (Figs. 3*a*–3*c*). The optimal concentration of IGF-I for stimulation of neuroblastoma cell proliferation was 25 ng/ml (Figs. 3*a*–3*c*). A lower growth response in response to IGF-I was observed in CHP134, which correlated with impaired IGF-I-mediated signaling (Figs. 3*c* and 4*d*). NVP-AEW541 completely blocked IGF-I-stimulated cell growth in the 3 cell lines under study (Figs. 3*a*–3*c*). Insulin failed to stimulate growth in SH-SY5Y, LAN1, CHP134 or WAC2 cells (Fig. 3*d*), although the IR was functional, as assessed by the activation of Akt/PKB in response to insulin in CHP134 cells (Fig. 4*e*). However, insulin failed to activate the S6K and MEK/Erk pathways in these 2 cell lines (data not shown).

To investigate the contribution of PI3K/Akt, mTOR/S6K and MEK/Erk to neuroblastoma cell proliferation, the activation of these signaling pathways in response to IGF-I was first studied in various cell lines. A NVP-AEW541-sensitive (SH-SY5Y) cell line and 2 cell lines with reduced sensitivity (WAC2, CHP134) were chosen. Akt/PKB was activated by IGF-I in SH-SY5Y and WAC2 cells and the response was inhibited by NVP-AEW541 at concentrations of 0.6–1.2 μM (Figs. 4*a* and 4*b*). IGF-I also activated Akt/PKB, S6K and Erk1/2 in SH-SY5Y cells (Fig. 4*c*), but only Akt/PKB in CHP134 cells (Fig. 4*d*). NVP-AEW541 impaired the IGF-I-stimulated responses in both cell lines (Figs. 4*c* and 4*d*). IGF-I also stimulated activation of Akt/PKB and Erk1/2 in other neuroblastoma cell lines, including LAN1 and WAC2 cells (data not shown). NVP-AEW541 inhibited insulin-stimulated activation of Akt/PKB in CHP134 cells (Fig. 4*e*). Tyrosine phosphorylation of

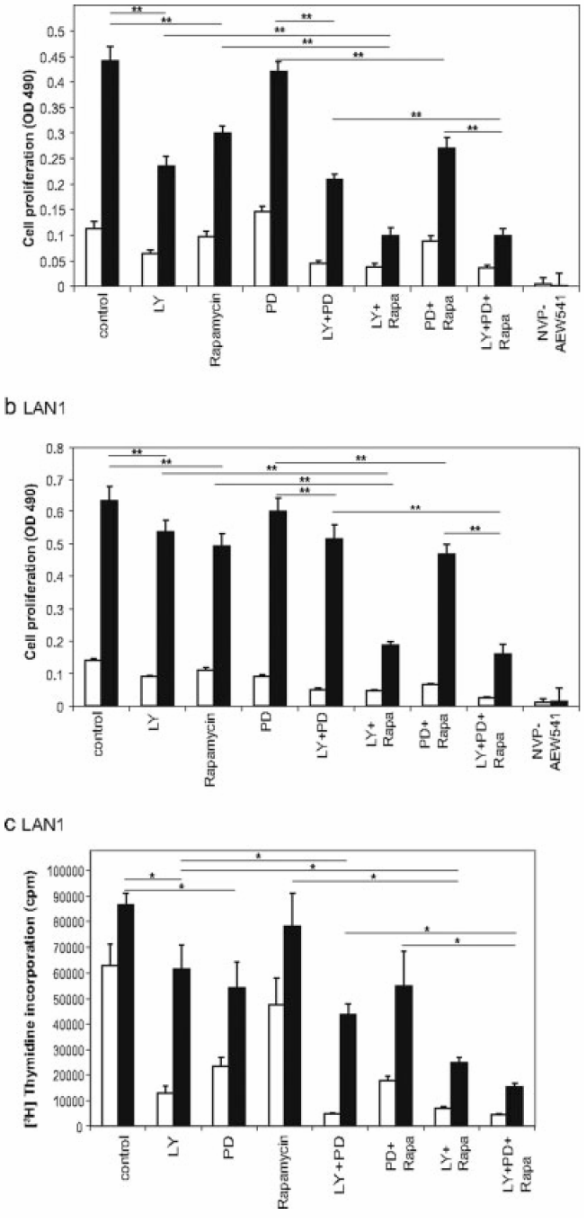

Figure 5 – Inhibition of IGF-I-stimulated neuroblastoma cell proliferation by a combination of PI3K inhibitor and rapamycin. (*a*), (*b*) SH-SY5Y (*a*) or LAN1 (*b*) cells were incubated with vehicle, LY294002 (10 μM), rapamycin (20 ng/ml), PD98059 (20 μM) as single agents or in combinations, or NVP-AEW541 (2.5 μM). Cells were stimulated with IGF-I (25 ng/ml) (dark grey bars) or left untreated (light grey bars) and cell proliferation measured after 72 hr. Data are mean with SD from 8 repetitions. (*c*) LAN1 cells were incubated with growth factors and inhibitors as in (*a*), (*b*). DNA synthesis was measured after 24 hr. Data are mean with SD from triplicate determinations. All the experiments were performed at least twice with similar results and 1 representative experiment is shown. * and ** indicate a significant difference between the indicated samples ($*p < 0.05$; $**p < 0.01$). Only the significant differences are shown.

the IGF-IR in response to its ligand was also impaired by NVP-AEW541 in SH-SY5Y cells, confirming the selectivity of the action of the inhibitor (Fig. 4*f*).

Contribution of the PI3K/PKB, mTOR/S6K and MEK/Erk pathways to IGF-I-stimulated cell growth in human neuroblastoma cells

Since our previous data had documented activation of PI3K/Akt, mTOR/S6K and MEK/Erk by IGF-I in neuroblastoma cells, we next investigated whether these pathways contributed to IGF-I-stimulated cell growth. SH-SY5Y or LAN1 cells were incubated with the PI3K inhibitor LY294002 (10 μM), rapamycin (20 ng/ml) or the MEK inhibitor PD98059 (20 μM) and cell growth in response to IGF-I (25 ng/ml) assessed after 72 hr. LY294002 and rapamycin significantly reduced IGF-I-stimulated cell growth in SH-SY5Y and in LAN1 cells (Figs. 5*a* and 5*b*). In contrast, PD98059 failed to significantly affect IGF-I-stimulated cell proliferation in both cell lines (Figs. 5*a* and 5*b*). The double combination of LY294002 and rapamycin was significantly more efficient than the single agents at inhibiting IGF-I-stimulated cell growth in SH-SY5Y cells (Fig. 5*a*). In LAN1 cells, LY294002 and rapamycin were also significantly more potent when used in combination with each other, than as single agents (Fig. 5*b*). The triple combination of LY294002, rapamycin and PD98059 was equivalent to the treatment with LY294002 and rapamycin in SH-SY5Y and LAN1 cells (Figs. 5*a* and 5*b*). The combination of LY294002, rapamycin and PD98059 was slightly less potent than NVP-AEW541 at inhibiting proliferation of SH-SY5Y and LAN1 cells (Figs. 5*a* and 5*b*).

To investigate whether the above results were caused by effects on cell proliferation, DNA synthesis experiments were performed in LAN1 cells under the same conditions. LY294002 and PD98059 significantly impaired IGF-I-stimulated DNA synthesis as single agents (Fig. 5*c*). The combination of LY294002 and rapamycin was significantly more efficient at impairing IGF-I-stimulated DNA synthesis than the single agents (Fig. 5*c*). The triple combination of LY294002, PD98059 and rapamycin did not have a significantly higher effect than the combination, of LY294002 and rapamycin, confirming the results obtained in the MTS assay (Fig. 5*b*).

NVP-AEW541 induces apoptosis and enhances the effects of cisplatin in neuroblastoma cells

To investigate whether induction of apoptosis contributed to the ability of NVP-AEW541 to inhibit neuroblastoma cell growth, the activation of caspase-3 and cleavage of PARP were analyzed in cells treated with the inhibitor. In LAN1 cells, increasing concentrations of NVP-AEW541 induced caspase-3 activation and PARP cleavage (Fig. 6*a*), demonstrating that the inhibitor causes apoptotic cell death in these neuroblastoma cells. Intriguingly, NVP-AEW541 failed to induce caspase-3 activation of PARP cleavage in the WAC2 cell line, which is comparatively more resistant to the inhibitor (data not shown). The ability of NVP-AEW541 to induce apoptosis in the panel of neuroblastoma cell lines was then investigated using an alternative assay measuring cytoplasmic histone-associated DNA fragments. The induction of apoptosis by NVP-AEW541 was highest in SH-SY5Y, LAN1 and NB69 cell lines (Fig. 6*b*). In contrast, WAC2 cells were comparatively more resistant to NVP-AEW541-induced apoptosis (Fig. 6*b*). In most cell lines, except NB69, the extent of NVP-AEW541-induced apoptosis (Fig. 6*b*) reflected the sensitivity of the cell line to the inhibitor (Fig. 2*d*).

Resistance of human neuroblastoma to chemotherapy is a frequent cause of treatment failure in this malignancy. Therefore, we next investigated whether inhibition of IGF-IR signaling by NVP-AEW541 could sensitize human neuroblastoma cells to the action of chemotherapeutic agents. Increasing concentrations of cisplatin resulted in effective suppression of cell growth in SH-SY5Y cells with an IC_{50} in the range of 0.1–1.0 μM (data not shown). SH-SY5Y cells were then exposed to cisplatin in the presence of increasing, but still non-toxic, concentrations of NVP-AEW541. At a concentration of cisplatin of 0.1 μM, NVP-AEW541 enhanced the effect of the chemotherapeutic drug on SH-SY5Y

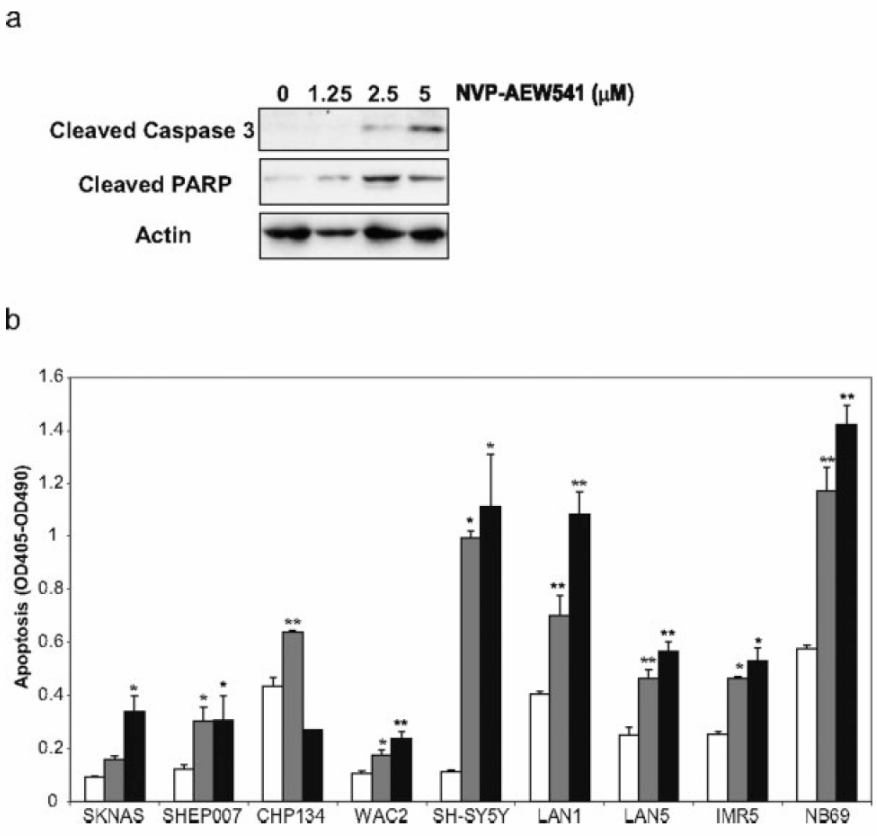

FIGURE 6 – NVP-AEW541 induces apoptosis in neuroblastoma cell lines. (*a*) LAN1 cells were incubated with increasing concentrations of NVP-AEW541 for 24 hr at 37°C. Cell lysates were analyzed by SDS-PAGE and Western blot for caspase-3, PARP, or actin. The bands corresponding to cleaved (active) caspase-3 (20 kDa) and cleaved PARP (85 kDa) are shown. (*b*) Neuroblastoma cell lines (as indicated) were incubated with vehicle (open bars) or NVP-AEW541 (1.25 μM, grey bars; 2.5 μM, black bars). Apoptosis was determined after 24 hr using the Cell Death Detection ELISA^{PLUS} assay. Data are mean with SD from triplicate determinations. All the experiments were performed at least twice with similar results and 1 representative experiment is shown. * and ** indicate a significant difference between the control and NVP-AEW541-treated samples (*$p < 0.05$; **$p < 0.01$).

cells (Fig. 7*a*). The effect of the combination of NVP-AEW541 (0.125 μM) and cisplatin (0.1 μM) on cell proliferation was about equivalent to the sum of the effects of the drugs used as single agents (71.6 % growth inhibition *versus* 61.3 %). Additive effects of cisplatin and NVP-AEW541 were also observed in CHP134 cells (Fig. 7*b*), but not in WAC2 cells (data not shown). We next investigated whether treatment of neuroblastoma cells with low doses of NVP-AEW541 and cisplatin resulted in increased apoptosis. Indeed, low doses of NVP-AEW541 combined with cisplatin induced increased apoptosis in the SH-SY5Y and LAN1 cell lines (Figs. 7*c* and 7*d*). Combining cisplatin at a concentration of 1.0 μM with NVP-AEW541 (1.2 μM) resulted in significantly enhanced apoptosis in both cell lines, as compared to the response to the single agents (Figs. 7*c* and 7*d*).

Activation of Akt/PKB decreases the sensitivity of neuroblastoma cells to NVP-AEW541

The comparative analysis of the panel of neuroblastoma cell lines had revealed a correlation between high levels of Akt/PKB activation and resistance to NVP-AEW541 in the WAC2 cell line (Figs. 1*b* and 2*a*). Therefore, we next investigated whether inhibiting Akt/PKB in WAC2 cells decreased the IC_{50} of the cells for the IGF-IR inhibitor. A pharmacological Akt inhibitor (1L-6-hydroxymethyl-*chiro*-inositol 2-(R)-2-*O*-methyl-3-*O*-octadecylcarbonate) was first titrated in the growth assay in medium containing a high (10%) or low (1%) serum concentration. No significant differences were observed in the growth rates of the WAC2 cells in either low- or high-serum-containing medium (Fig. 8*a*). Increasing concentrations of the Akt/PKB inhibitor did not significantly affect cell proliferation in high-serum-containing medium, but complete inhibition of cell growth was observed at a 20 μM concentration of the inhibitor in low-serum-containing medium (Fig. 8*a*). The ability of the Akt/PKB inhibitor to block the activation of the kinase in WAC2 cells was confirmed by Western blot analysis (Fig. 8*b*). To confirm the data obtained with the pharmacological inhibitor, WAC2 cells were transiently transfected with a catalytically inactive dominant negative Akt/PKB mutant. Proliferation of WAC2 cells was almost completely inhibited upon transfection of the dominant negative Akt/PKB construct (Fig. 8*c*).

WAC2 cells were then incubated with the pharmacological Akt/PKB inhibitor (20 μM) in the presence of increasing concentrations of NVP-AEW541 in high-serum-containing medium. The Akt/PKB inhibitor effectively increased the sensitivity of WAC2 cells to the IGF-IR inhibitor (Fig. 8*d*), confirming the model that enhanced Akt/PKB activation can result in resistance to NVP-AEW541.

To further support the model above, SH-SY5Y cells were transfected with a constitutively activated mutant of Akt/PKB and the sensitivity of the cells to NVP-AEW541 was assessed in the proliferation assay. Western blot analysis confirmed expression of transfected myristoylated catalytically active Akt/PKB in SH-SY5Y cells (data not shown). The active Akt did not significantly stimulate SH-SY5Y cell growth in high-serum-containing medium (data not shown). However, SH-SY5Y cells transfected with activated Akt were significantly less sensitive to NVP-AEW541 than cells transfected with the empty expression vector (Fig. 8*e*).

Impact of the expression of activated S6K on neuroblastoma sensitivity to IGF-IR inhibitors

The comparative Western blot analysis had revealed high levels of S6 protein phosphorylation in some neuroblastoma cell lines with intermediate or low sensitivities to NVP-AEW541, such as SKNAS, SHEP007, CHP134, WAC2 and NB69

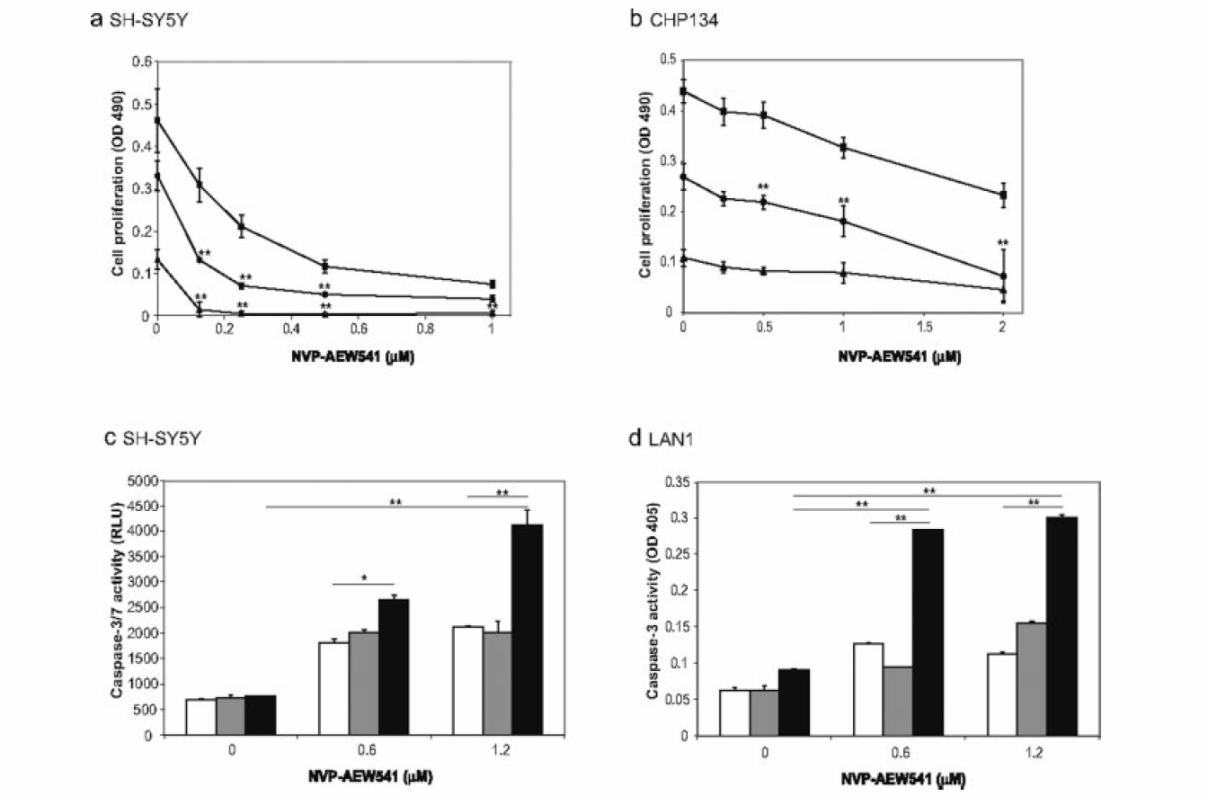

FIGURE 7 – NVP-AEW541 sensitizes neuroblastoma cells to cisplatin. (*a*), (*b*) SH-SY5Y (*a*) or CHP134 (*b*) cells were incubated with increasing concentrations of NVP-AEW541 in the absence (squares) or presence of cisplatin (circles: 0.1 μM (*a*), 2.5 μM (*b*); triangles: 1.0 μM (*a*), 5 μM (*b*)). Cell growth was measured after 72 hr. Data are mean with SD from 8 repetitions. (*c*), (*d*) SH-SY5Y (*c*) or LAN1 (*d*) cells were incubated with increasing concentrations of NVP-AEW541 for 24 hr at 37°C in the absence (open bars) or presence of cisplatin (0.1 μM, grey bars; 1.0 μM, closed bars). Cell lysates were analyzed for caspase activity using a luminometric (*c*) or colorimetric (*d*) assay. All the experiments were performed at least twice with similar results and 1 representative experiment is shown. * and ** indicate a significant difference between the samples treated with the combination of the 2 drugs compared to single drug treatments (*$p < 0.05$; **$p < 0.01$).

(Fig. 1*b*). To investigate whether activation of the S6K pathway could reverse the sensitivity of neuroblastoma cells to NVP-AEW541, SH-SY5Y cells were transfected with a constitutively activated mutant of S6K1. Western blot analysis confirmed increased phosphorylation of the S6 protein in the S6K1-transfected cells (Fig. 9*a*). The active S6K1 stimulated SH-SY5Y cell growth in serum-containing medium (Fig. 9*b*). The sensitivity of the stably transfected cells to NVP-AEW541 was then compared to that of cells transfected with an empty vector in the proliferation assay. SH-SY5Y cells transfected with activated S6K1 were less sensitive to NVP-AEW541 than cells transfected with the empty expression vector (Fig. 9*c*). The difference in sensitivities between the S6K1- and vector-transfected SH-SY5Y cells (IC_{50} 2.0 *vs.* 0.3 μM) was less marked than in the case of activated Akt (IC_{50} 7.0 *vs.* 0.3 μM) (Fig. 8*e*).

Discussion

Pharmacological inhibitors targeting the tyrosine kinase activity of RTKs have been extensively studied as antitumor agents, such as in the case of the EGFR.[23] Recent reports have highlighted that differences in the sensitivities of human tumor cells to such agents can be caused by mutations in the receptor, since lung cancer cells with activating EGFR mutations were more sensitive to the action of the inhibitor gefitinib.[24,25]

Human neuroblastoma cells have been reported to express a variety of RTKs, and IGF signaling has been demonstrated to control tumor cell proliferation, motility and survival.[5–7] Neutralizing antibodies directed against the IGF-IR were previously shown to inhibit the proliferation of neuroblastoma cells.[26] Given the recent availability of the specific IGF-IR kinase inhibitor NVP-AEW541,[21] we investigated the potential of this new compound as an antiproliferative agent in human neuroblastoma cells lines. NVP-AEW541 inhibited proliferation of human neuroblastoma cell lines, as assessed by 3 different assays. The ability of the inhibitor to block neuroblastoma cell growth was a result of both its inhibitory effects on DNA synthesis and induction of apoptosis. Surprisingly, there were significant variations in the sensitivities of 9 different neuroblastoma cell lines to NVP-AEW541, with only 2 cell lines (SH-SY5Y and LAN1) displaying IC_{50}s compatible with inhibition of IGF-IR signaling alone.[21] The decreased sensitivities of some of the neuroblastoma cell lines (CHP134 and IMR5) to NVP-AEW541 could be explained by high expression levels of the IR, since the inhibitor also blocks the kinase activity of this receptor with an IC_{50} of 2.3 μM *in vivo*.[21] The observation that insulin activates Akt/PKB in neuroblastoma cells indicates that the IR provides a survival signal that blocks apoptosis induced by IGF-IR inhibition. This may be relevant for the growth experiments performed in medium containing FCS, since serum contains insulin in addition to IGF-I and IGF-II. Intriguingly, high activation of Akt/PKB also correlated with resistance to NVP-AEW541 in other neuroblastoma cell lines, such as WAC2. The resistance of WAC2 cells to NVP-AEW541 was not caused by the fact that this particular cell

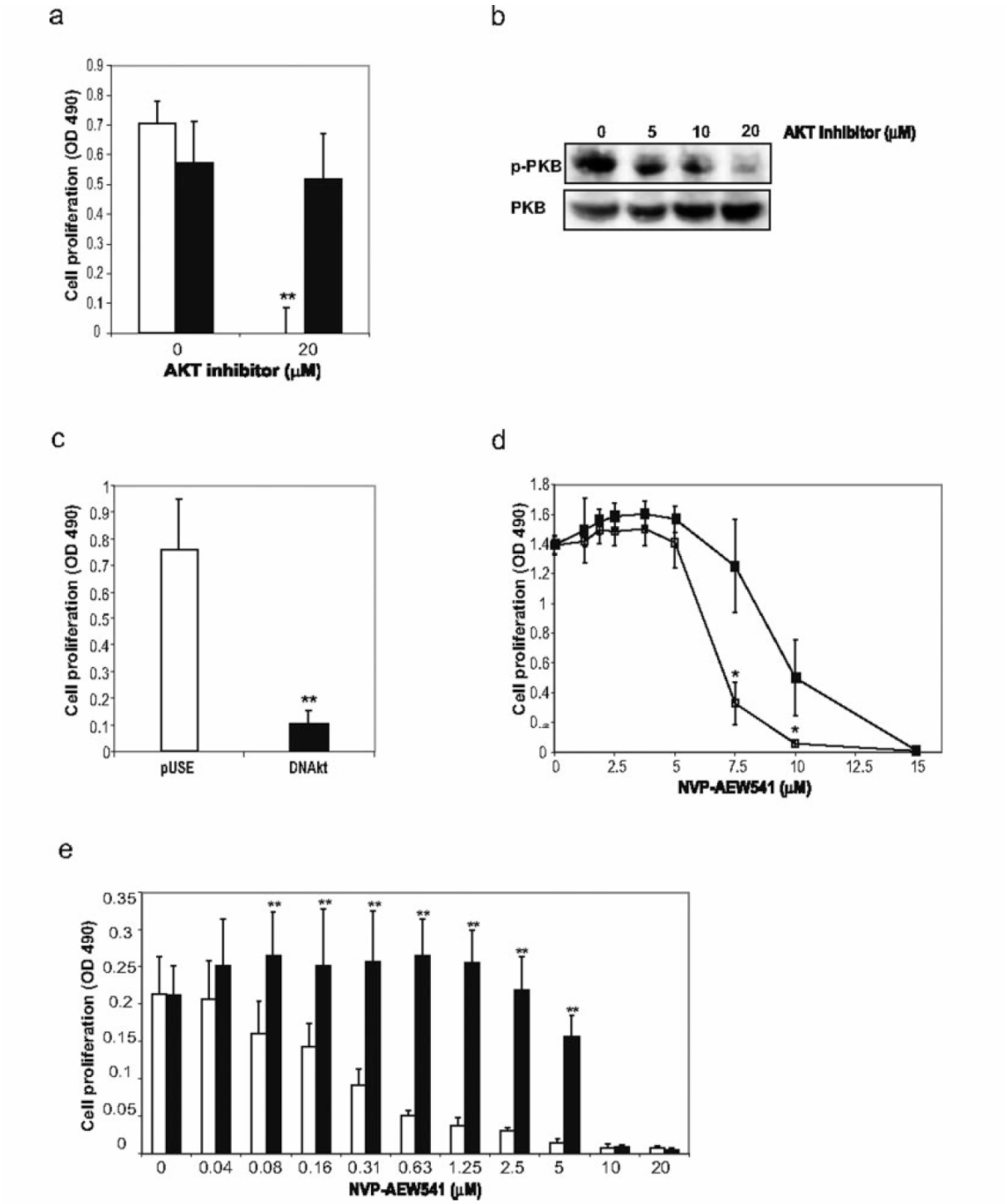

Figure 8 – Akt/PKB modulates the sensitivity of neuroblastoma cells to NVP-AEW541. (*a*) WAC2 cells were incubated with vehicle or an Akt/PKB inhibitor (20 μM) in medium containing 1% (open bars) or 10% FCS (closed bars). Cell proliferation was measured after 72 hr using an MTS assay. Data are mean with SD from 8 repetitions. (*b*) WAC2 cells were incubated for 18 hr with increasing concentrations of Akt inhibitor. Cell lysates were analyzed by SDS-PAGE and Western blot for activated Akt/PKB (p-PKB) or total Akt/PKB. (*c*) WAC2 cells were transiently transfected with dominant negative Akt (DNAkt) or empty vector (pUSE). Cell proliferation in medium containing 10% FCS was measured as in (*a*). (*d*) WAC2 cells were incubated with increasing concentrations of NVP-AEW541 in the presence of vehicle (closed squares) or Akt/PKB inhibitor (20 μM, open squares) in medium containing 10% FCS. Cell proliferation was measured as in (*a*). (*e*) SH-SY5Y were stably transfected with active myristoylated Akt/PKB (closed bars) or empty vector (pUSE, open bars). Cell proliferation in the presence of increasing concentrations of NVP-AEW541 was measured as in (*a*). Data are mean with SD from 8 repetitions. All the experiments were performed at least twice with similar results and 1 representative experiment is shown. * and ** indicate a significant difference between control and the inhibitor-treated samples, or between the activated Akt transfectants and the vector-transfected cells (*$p < 0.05$; **$p < 0.01$).

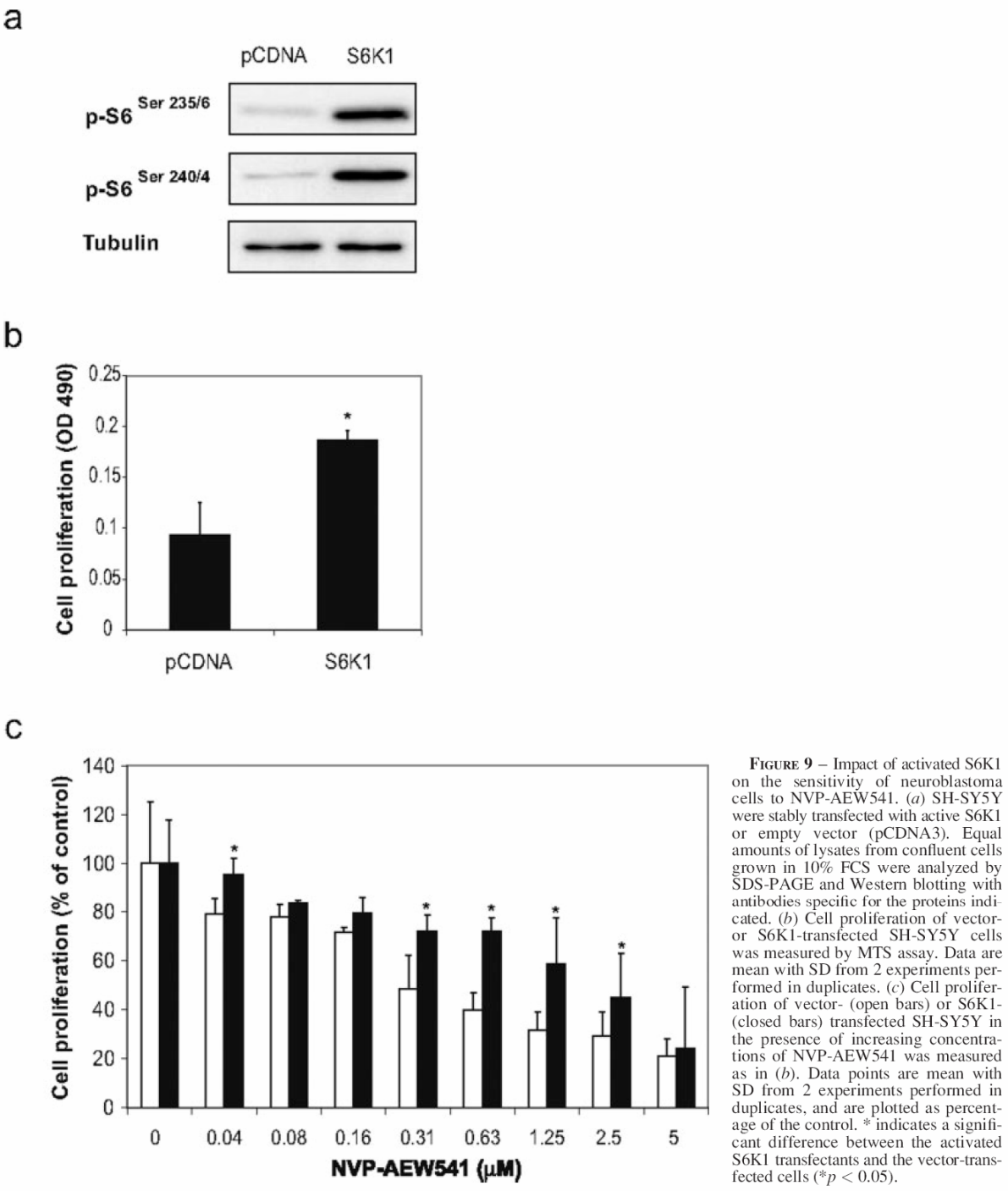

FIGURE 9 – Impact of activated S6K1 on the sensitivity of neuroblastoma cells to NVP-AEW541. (*a*) SH-SY5Y were stably transfected with active S6K1 or empty vector (pCDNA3). Equal amounts of lysates from confluent cells grown in 10% FCS were analyzed by SDS-PAGE and Western blotting with antibodies specific for the proteins indicated. (*b*) Cell proliferation of vector- or S6K1-transfected SH-SY5Y cells was measured by MTS assay. Data are mean with SD from 2 experiments performed in duplicates. (*c*) Cell proliferation of vector- (open bars) or S6K1- (closed bars) transfected SH-SY5Y in the presence of increasing concentrations of NVP-AEW541 was measured as in (*b*). Data points are mean with SD from 2 experiments performed in duplicates, and are plotted as percentage of the control. * indicates a significant difference between the activated S6K1 transfectants and the vector-transfected cells (*$p < 0.05$).

line expresses a non-functional IGF-IR, since IGF-I-stimulated signaling could be observed in these cells. Our data convincingly demonstrate that Akt/PKB activation renders neuroblastoma cells less sensitive to IGF-IR inhibition. Indeed, a pharmacological Akt/PKB inhibitor could reverse the resistance of the WAC2 cell line, and transfection of activated Akt into a NVP-AEW541-sensitive cell line decreased the sensitivity of the cells to the IGF-IR inhibitor. The simplest model to account for these observations is that activation of Akt/PKB suppresses the antiproliferative and proapoptotic signals induced by the IGF-IR inhibitors, for example by blocking caspase activation. The molecular mechanism of Akt/PKB activation in the WAC2 cell line is currently unclear. Decreased PTEN expression was not observed in this cell line, as compared to NVP-AEW541-sensitive cell lines.

Other potential mechanisms of Akt/PKB activation include overexpression of other RTKs or PI3K, or mutations in the *PIK3CA* gene encoding p110α. A recent report described decreased sensitivity of small cell lung cancer cells to the IGF-IR inhibitor NVP-ADW742 due to coexpression of the IGF-IR and c-Kit.[27] Our preliminary experiments did not reveal any significant differences in RTKs or PI3K expression between WAC2 cells and other cell lines, which may account for the enhanced Akt/PKB activation. However, activation of Akt was observed in WAC2 cells only when grown in the presence of serum. This may be the consequence of either activation of another receptor by a growth factor present in the serum, or the fact that, even when activated by mutation or *PTEN* deletion, PI3K still requires translocation to the membrane through receptor association for full activation.

An additional mechanism of resistance to NVP-AEW541 in neuroblastoma cell lines appeared to be activation of the S6K pathway. Indeed, cell lines displaying high levels of S6 protein phosphorylation (including SKNAS, SHP007 and NB69) were less sensitive to NVP-AEW541 than were SH-SY5Y and LAN1, where the levels of S6 protein phosphorylation were low. In addition, transfection of an activated S6K1 mutant into SH-SY5Y reduced the sensitivity of the cells to the IGF-IR inhibitor. Thus, while NVP-AEW541 impairs activation of Akt and S6K in response to IGF-I, resulting in reduced neuroblastoma cell growth, activation of these downstream signaling pathways appears to result in resistance of the cells to NVP-AEW541.

Our observations may have important implications for the future design of *in vivo* experiments aimed at validating IGF-IR inhibitors for clinical trials in human neuroblastoma. Indeed, expression of the IGF-IR alone did not appear to predict sensitivity to NVP-AEW541, or anti-IGF-IR neutralizing antibody, in a panel of neuroblastoma cell lines that are relevant for the human disease. A detailed analysis of the expression levels of the IGF-IR, IR, activated Akt/PKB and S6K in the tumors may be required before designing *in vivo* experiments involving these agents in human neuroblastoma. Intriguingly, Akt/PKB phosphorylation was reported to correlate with gefitinib efficacy in nonsmall cell lung cancer.[28] The mechanism underlying these observations is thought to be that activating mutations in the EGFR cause increased antiapoptotic signaling in the lung cancer cells.[25] Since the tumor cells are thought to be then more dependent on these pathways, the action of gefitinib is enhanced.[25] It remains, however, unclear, whether in the case of Akt/PKB activation by other mechanisms, such as *PTEN* loss, PI3K amplification or mutations, the same responses would be observed.

The present report also provides new information about the molecular mechanisms involved in IGF-I-stimulated cell proliferation in neuroblastoma cell lines. Optimal growth responses in response to IGF-I were observed in cells displaying activation of Akt/PKB, S6K and Erk1/2 (SH-SY5Y), while in cells where S6K and Erk activation were impaired (CHP134), IGF-I-stimulated growth was significantly lower. The molecular mechanisms underlying these previously unreported differences in IGF-I-induced signaling are at present unclear. A possible explanation is that neuroblastoma cells with impaired Erk and S6K activation are missing expression of a critical component (adaptor molecule or kinase) that transmits IGF-IR signals to these downstream effectors. One of the potential implications of these findings is that, in human neuroblastoma, the IGF-IR may not always promote cell growth, depending on whether all of its signaling components are functionally expressed.

Pharmacological inhibitors of either PI3K or mTOR, but not MEK/Erk, significantly impaired IGF-I-stimulated growth, when used as single agents in SH-SY5Y or LAN1 cells. Blocking PI3K and mTOR signaling by combining LY294002 and rapamycin resulted in the most efficient inhibition of IGF-I-stimulated proliferation, as compared to the inhibition by other combinations of 2 pharmacological inhibitors. Interestingly, insulin was a poor stimulator of neuroblastoma cell growth, in comparison to IGF-I, which correlated with its inability to activate S6K and Erk1/2. Although the MEK inhibitor PD98059 failed to significantly inhibit IGF-I-stimulated cell growth as a single agent, it inhibited basal growth of several neuroblastoma cell lines in serum-containing medium (unpublished observations). This indicates that, in contrast to other RTKs, the IGF-IR may be less dependent on MEK/Erk for its growth signaling.

The data presented here also highlight a potential use for NVP-AEW541 in combination with chemotherapeutic agents such as cisplatin in human neuroblastoma. Similar results were recently reported with the related IGF-IR inhibitor NVP-ADW742 in combination with etoposide or carboplatin in small cell lung cancer.[29] Since resistance to chemotherapy is a frequent cause of treatment failure and death in neuroblastoma, combinations of IGF-IR inhibitors with different chemotherapeutic agents may, in the future, be considered for the treatment of neuroblastoma.

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3.2.3 Novel role for insulin as an autocrine growth factor for malignant brain tumour cells

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Novel role for insulin as an autocrine growth factor for malignant brain tumour cells

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AT/RTs (atypical teratoid/rhabdoid tumours) of the CNS (central nervous system) are childhood malignancies associated with poor survival rates due to resistance to conventional treatments such as chemotherapy. We characterized a panel of human AT/RT and MRT (malignant rhabdoid tumour) cell lines for expression of RTKs (receptor tyrosine kinases) and their involvement in tumour growth and survival. When compared with normal brain tissue, AT/RT cell lines overexpressed the IR (insulin receptor) and the IGFIR (insulin-like growth factor-I receptor). Moreover, insulin was secreted by AT/RT cells grown in serum-free medium. Insulin potently activated Akt (also called protein kinase B) in AT/RT cells, as compared with other growth factors, such as epidermal growth factor. Pharmacological inhibitors, neutralizing antibodies, or RNAi (RNA interference) targeting the IR impaired the growth of AT/RT cell lines and induced apoptosis. Inhibitors of the PI3K (phosphoinositide 3-kinase)/Akt pathway also impaired basal and insulin-stimulated AT/RT cell proliferation. Experiments using RNAi and isoform-specific pharmacological inhibitors established a key role for the class I_A PI3K p110α isoform in AT/RT cell growth and insulin signalling. Taken together, our results reveal a novel role for autocrine signalling by insulin and the IR in growth and survival of malignant human CNS tumour cells via the PI3K/Akt pathway.

Key words: Akt, atypical teratoid/rhabdoid tumour (AT/RT), cell proliferation, central nervous system, insulin-like growth factor (IGF), phosphoinositide 3-kinase (PI3K).

INTRODUCTION

The AT/RT (atypical teratoid/rhabdoid tumour) is a highly malignant paediatric tumour of the CNS (central nervous system), which is characterized by unique clinical, biological and histological features [1–4]. Patients with CNS AT/RT respond very poorly to chemotherapy and radiotherapy and thus the prognosis for this particular malignancy is very poor [4–6]. AT/RT is related to other rhabdoid tumours of the kidney [MRT (malignant rhabdoid tumour)] and soft tissues. Most of the rhabdoid tumours harbour inactivating biallelic alterations in the *hSNF5* (Human *SNF5*)/*INI1* gene, a tumour suppressor gene on chromosome 22 [7–9]. hSNF5/INI1 is a component of the ATP-dependent chromatin remodelling SWI/SNF complex [10]. There are multiple sets of mammalian SWI/SNF complexes, with varying subunit compositions, which play important roles in transcriptional regulation, through both activation and repression of gene transcription [10–12]. Mice with a targeted disruption of the *INI1* gene developed tumours at a high frequency and the resulting tumours displayed loss of expression of the hSNF5/INI1 protein [13,14]. Deletion of *hSNF5/INI1* was recently reported to cooperate with p53 loss in oncogenic transformation in murine models [15,16]. One of the mechanisms by which *hSNF5/INI1* exerts its tumour suppressor function was shown to involve repression of cyclin D1 gene expression [17]. Targeting cyclin D1 gene expression was thus suggested to represent a novel therapeutic strategy for AT/RT [17,18].

The insulin/IGF (insulin-like growth factor) family of growth factors are part of an evolutionarily conserved signalling system with a critical role in the growth and development of many tissues as well as the regulation of overall growth and metabolism. This signalling system is characterized by a high complexity and involves multiple proteins including three receptors [IR (insulin receptor), IGFIR (IGF-I receptor) and IGF-II/M-6-PR (mannose 6-phosphate receptor)], three ligands (insulin, IGF-I and IGF-II) and six known types of circulating binding proteins [IGFBP1 (IGF-binding protein 1)–IGFBP6] [19,20]. Both IGF-I and IGF-II bind to the IGFIR, although IGF-I shows a higher affinity than IGF-II [20]. Insulin, the main ligand for the IR, has an IGFIR-binding affinity that is much lower than that of IGF-I [19,20]. The specific receptor for IGF-II, the M-6-PR, differs significantly from the IGFIR, possesses no tyrosine kinase activity and was reported to target IGF-II for lysosomal degradation [21,22]. Signalling by the IGFIR plays a fundamental role in cell growth and malignant transformation and is an important inhibitor of apoptosis [23,24]. The IGFIR is overexpressed in a variety of human tumours including malignant brain tumours [25]. Decreased receptor expression or impaired function was reported to induce a reversal of the transformed phenotype, apoptosis and a decrease in cellular radioresistance and chemoresistance [26]. The IGFIR has thus

Abbreviations used: AT/RT, atypical teratoid/rhabdoid tumour; CNS, central nervous system; Cy3, indocarbocyanine; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGF, epidermal growth factor; EGFR, EGF receptor; ERK1/2, extracellular-signal-regulated kinase 1/2; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, haemagglutinin; hSNF5, human SNF5; IGF, insulin-like growth factor; IGFBP1, IGF-binding protein 1; IGFIR, IGF-I receptor; IR, insulin receptor; M-6-PR, mannose 6-phosphate receptor; MRT, malignant rhabdoid tumour; mTOR, mammalian target of rapamycin; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; PDX-1, pancreatic duodenal homeobox-1; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PTEN, phosphatase and tensin homologue deleted on chromosome 10; RNAi, RNA interference; RTKs, receptor tyrosine kinases; RT, reverse transcriptase; SCF, stem cell factor; shRNA, small-hairpin RNA; siRNAs, small interfering RNAs; S6K, S6 kinase; Tos-Lys-CH_2Cl, tosyl-lysylchloromethane.

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been proposed to be a target for the development of novel anti-cancer therapies [26–29]. In medulloblastoma, targeting of the IGFIR with the inhibitor NVP-AEW541 [30] was recently shown to impair cell growth and survival [31]. In AT/RT cells, a recent report has shown that the IGFIR is involved in anti-apoptotic signalling and contributes to chemoresistance [32]. Less is known about the potential involvement of the related IR in human cancer, although its role has been described in the pathogenesis of certain malignancies [33].

A critical intracellular signalling mediator of the IGFIR is the PI3K (phosphoinositide 3-kinase)/Akt [also called PKB (protein kinase B)] pathway [27,34,35]. Indeed, PI3K signalling is implicated in the control of cell proliferation, survival and motility/metastasis downstream of many different growth factor receptors [35,36]. The importance of PI3K signalling in human cancer is highlighted by the fact that mutations in the tumour suppressor gene *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) occur frequently in human tumours [36,37]. PTEN is a phosphatase that antagonizes the action of PI3K by dephosphorylating the D-3 position of polyphosphoinositides [38]. Moreover, previous reports have described activating mutations in the *PIK3CA* gene encoding the catalytic p110α isoform of class I_A PI3K in a variety of human cancers, including, breast, colon and ovarian cancers, as well as medulloblastoma [39,40].

In the present study, we have investigated the expression pattern and biological functions of components of the IR and IGFIR signalling system in human AT/RT and MRT cell lines. Moreover, we have evaluated the potential of targeting the IR or the IGFIR using RNAi (RNA interference), neutralizing antibodies or the inhibitor NVP-AEW541 [30] as an antiproliferative approach in AT/RT cells. Finally, we have investigated whether targeting downstream signalling mediators of the IR could suppress growth and induce apoptosis in AT/RT cell lines. Our findings describe for the first time a role for autocrine signalling by insulin and the IR in growth and survival of AT/RT cells, which involves the PI3K/Akt pathway.

MATERIALS AND METHODS

Reagents and antibodies

Antibodies against Akt/PKB, caspase 3, HA (haemagglutinin) epitope tag, ERK1/2 (extracellular-signal-regulated kinase 1/2), IGFIRβ, INI1, IRβ, lamin B, p110β and p110δ were obtained from Santa Cruz Biotechnology. Antibodies against activated ERK1/2 (Thr^{202}/Tyr^{204}), activated Akt (Ser^{473}), and Thr^{389}-phosphorylated S6K (S6 kinase) were from Cell Signaling Technology. The Akt inhibitor [1L-6-hydroxymethyl-chiro-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate], IGF-I, the IGFIR neutralizing antibody (clone αIR3), LY294002, rapamycin and PD98059 were from Calbiochem. The anti-β-tubulin antibody and insulin were from Sigma–Aldrich. The IR neutralizing antibody (clone 47-9) was from Biosource. The anti-p85α antibody was from Upstate Biotechnology. The antibody against p110α (clone U3A) was a generous gift from Dr A. Klippel (atugen, Berlin, Germany). NVP-AEW541 (Novartis Pharma), YM024, TGX-221 and IC87114 (ICOS Corporation) were dissolved in DMSO at 10 mM and diluted into cell culture medium just prior to use.

Cell lines and cell culture

The human MRT cell lines AS, LP, MON and STM [9] have been described previously. The DAOY medulloblastoma cell line was purchased from the A.T.C.C. (Rockville, MD, U.S.A.). The BT-12 and BT-16 human CNS AT/RT cell lines [32,41] were gifts from Dr Jaclyn Biegel (The Children's Hospital of Philadelphia, PA, U.S.A.). These cell lines have been established from two infants with CNS AT/RT (BT-12 from a 6-week-old female; BT-16 from a 2-year-old male). They have been analysed for *INI1* mutations by Dr Jaclyn Biegel and both contain *INI1* mutations. Human AT/RT cell lines were grown in DMEM (Dulbecco's modified Eagle's medium; Life Technologies/Invitrogen) with 10 % (v/v) FCS (foetal calf serum) and penicillin/streptomycin/L-glutamine, and passaged every 3–5 days by trypsinization. Human MRT cell lines were grown in RPMI 1640 (Life Technologies/Invitrogen) with 10 % FCS and penicillin/streptomycin/L-glutamine. For serum-starving, the cells were incubated for 16 h in DMEM containing 0.5 % FCS. All cell cultures were maintained at 37 °C in a humidified atmosphere with 5 % CO_2.

Cell proliferation and apoptosis

AT/RT cell lines (10^5/ml) were grown in 96-well plates for 3 days in serum (1 or 10 %)-containing medium in the presence or absence of inhibitors. For growth factor stimulations, cells were incubated in medium containing 1 % FCS. Cell proliferation was analysed by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] assay using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega).

For detection of apoptosis, AT/RT cells [1×10^6 cells/well in 6-well plates (35 mm)] were incubated for 24 h in the presence or absence of inhibitors. The cells were then washed in 1× PBS and lysed in 1× gel-loading buffer [50 mM Tris/HCl, pH 6.8, 2 % (w/v) SDS, 100 mM DTT (dithiothreitol), 0.1 % (w/v) Bromophenol Blue and 10 % (w/v) glycerol]. The samples were denatured for 3 min at 100 °C and analysed by SDS/PAGE and Western blot with anti-caspase 3 antibodies.

ELISAs

AT/RT cells were seeded at 5×10^4 cells/well in 6-well plates (35 mm). After 3 h, the cells were washed three times in serum-free medium and incubated in 2 ml of DMEM containing penicillin/streptomycin/L-glutamine. After 5 days, the supernatants were collected, centrifuged to remove cellular contaminants and human insulin was quantified by using the AIA-PACK IRI immunoenzymometric assay (Tosoh Corporation). The background signal caused by the cell culture medium in the absence of cells was subtracted from the measured values.

Immunofluorescence

AT/RT cells were grown on coverslips and serum-starved for 24 h. The cells were then fixed with 4 % (w/v) paraformaldehyde in 1× PBS and permeabilized with methanol for 10 min at −20 °C. The coverslips were blocked with 0.5 % (w/v) non-fat dry milk in 1× PBS for 1 h and incubated with anti-insulin antibody (1:50; Dako) or anti-IRβ (1:100; Santa Cruz Biotechnology) for 1 h. The detection was performed using secondary anti-guinea-pig antibody coupled with FITC, or anti-rabbit antibody coupled with Cy3 (indocarbocyanine). Pictures were taken at ×100 magnification with a Zeiss Axioskop fluorescence microscope.

Immunoprecipitations

AT/RT cells grown to confluence on 10 cm dishes were serum-starved and stimulated with growth factors for 10 min at 37 °C. After washing with ice-cold 1× PBS, the cells were lysed for 20 min on ice in 1 ml of lysis buffer [20 mM Hepes/NaOH,

pH 7.4, 150 mM NaCl, 1 % (w/v) Triton X-100, 2 mM EDTA, 10 mM sodium fluoride, 10 % (w/v) glycerol, 1 mM PMSF, 5 mM benzamidine, 1 mM Tos-Lys-CH_2Cl (tosyl-lysylchloromethane), 10 μM leupeptin, 10 μM pepstatin A, 2 mM Na_3VO_4 (sodium orthovanadate), 10 mM 2-glycerophosphate and 10 mM NaF]. Immunoprecipitation was performed for 2 h at 4 °C with primary antibodies (diluted according to the manufacturer's instructions). Protein G–Sepharose CL-4B (Amersham Biosciences) was then added, and the incubation was continued for 1 h at 4 °C. The immunoprecipitates were washed three times in lysis buffer and resuspended in 1× gel-loading buffer. The samples were denatured for 3 min at 100 °C and analysed by SDS/PAGE and Western blot.

MS analysis

Samples (10 μl) were desalted using Millipore C18 ZipTips. An aliqust of 0.5 μl was applied directly to a prespotted AnchorChip™ target. After 3 min of incubation, 7 μl of washing solution (0.1 % trifluoroacetic acid) was added on the analyte solution and the whole droplet was removed after a few seconds. MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) mass spectra were acquired in linear mode using a Bruker Daltonics Autoflex™ mass spectrometer.

RT (reverse transcriptase)–PCR

Total cellular RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. For each RT–PCR, 2 μg of total RNA was used with the Qiagen One-Step RT–PCR kit. The following primers were used: insulin primer, sense 5′-CGTTCCCCGCACACTAGGTT-3′ and antisense 5′-GCAGCCTTTGTGAACCAACAC-3′; IGF-I primer, sense 5′-GTGCTGCTTTTGTGATTTCTT-3′ and antisense 5′-GTCTTGGGCATGTCGGTGTGG-3′; IGF-II primer, sense 5′-ATGGGGAAGTCGATGCTGGTG-3′ and antisense 5′-ACGGGGTATCTGGGGAAGTTG-3′; PDX-1 (pancreatic duodenal homeobox-1) primer, sense 5′-CTGCGGAGCCGGAGGAGAAC-3′ and antisense 5′-TCTAGAACTACACAGAGAGC-3′; GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primer, sense 5′-GAAGGTGAAGGTCGGAGTC-3′ and antisense 5′-GAAGATGGTGATGGGATTTC-3′. The reaction conditions were as follows: reverse transcription at 50 °C for 30 min, initial PCR activation at 95 °C for 15 min followed by 40 cycles at 95 °C for 15 s, 55 °C for 20 s and 72 °C for 40 s. The PCR products were analysed in 3 % (w/v) agarose gel.

SDS/PAGE and Western-blot analysis

Cellular lysates were prepared with lysis buffer [50 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 % Triton X-100, 1 % (w/v) deoxycholic acid, 0.1 % SDS, 10 % glycerol, 10 μM leupeptin, 10 μM pepstatin A, 1 mM PMSF, 5 mM iodoacetamide, 5 mM benzamidine, 1 mM Tos-Lys-CH_2Cl, 2 mM Na_3VO_4, 10 mM 2-glycerophosphate and 10 mM NaF]. The cells were lysed for 20 min on ice, scraped and lysates were centrifuged at 12000 ***g*** for 20 min at 4 °C. The supernatants were collected and normalized for protein content by using the BCA (bicinchoninic acid) protein assay kit (Pierce). One volume of 2× gel-loading buffer [100 mM Tris/HCl, pH 6.8, 4 % SDS, 200 mM DTT, 0.2 % (w/v) Bromophenol Blue and 20 % (w/v) glycerol] was then added to the samples, followed by denaturation for 3 min at 100 °C and analysed by SDS/PAGE. The gels were transferred on to a hydrophobic PVDF membrane (Hybond-P; Amersham Biosciences) by electrophoresis. The membranes were then blocked in 1× PBS, 5 % (w/v) BSA (phospho-specific antibodies) or 1× PBS/3 % non-fat dry milk (all other antibodies) for 16 h at 4 °C. The membranes were incubated with primary antibodies (diluted according to the manufacturer's protocol) for 16 h at 4 °C. After washing in 1× PBS and 0.1 % (w/v) Tween 20, the immunoblots were incubated with donkey anti-rabbit IgG or sheep anti-mouse IgG secondary antibodies (1:10000 dilution) coupled with horseradish peroxidase (Amersham Biosciences) for 1 h at room temperature. After washing of the immunoblots, chemiluminescence was used for detection, using the ECL® (enhanced chemiluminescence) Western blotting detection reagents (Amersham Biosciences) according to the manufacturer's protocol.

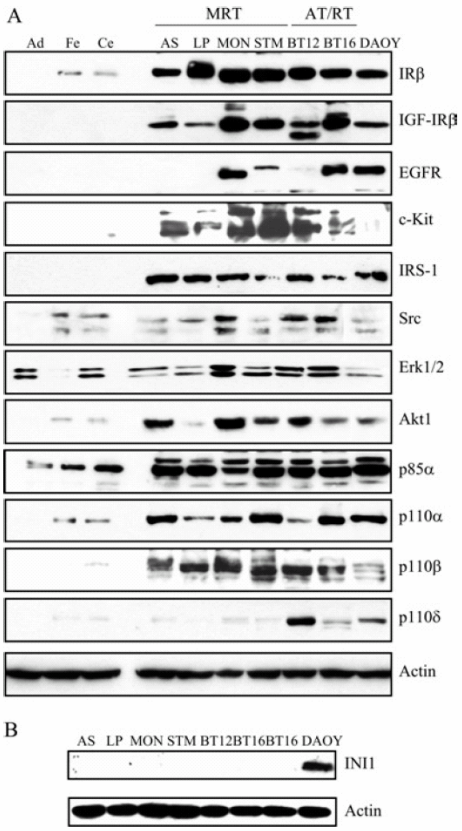

Figure 1 Expression of the IR and its downstream signalling components in MRT and AT/RT cells

(**A**) Equal amounts of lysates from adult brain (Ad), foetal brain (Fe), foetal cerebellum (Ce), or the cell lines indicated were analysed by Western blot for expression of the proteins indicated. The cell lines analysed were AS, LP, MON, STM (MRT), BT-12, BT-16 (AT/RT) and DAOY (medulloblastoma). (**B**) Equal amounts of lysates from the cell lines indicated were analysed by Western blot for expression of INI1.

Transient and stable transfections in AT/RT cells

Human AT/RT cells were transiently transfected with Akt/PKB constructs in pUSE (Upstate Biotechnology), or with PI3K shRNA (small-hairpin RNA) constructs in pRETRO-SUPER [42] by using Lipofectamine™ 2000 (Invitrogen). The transfections

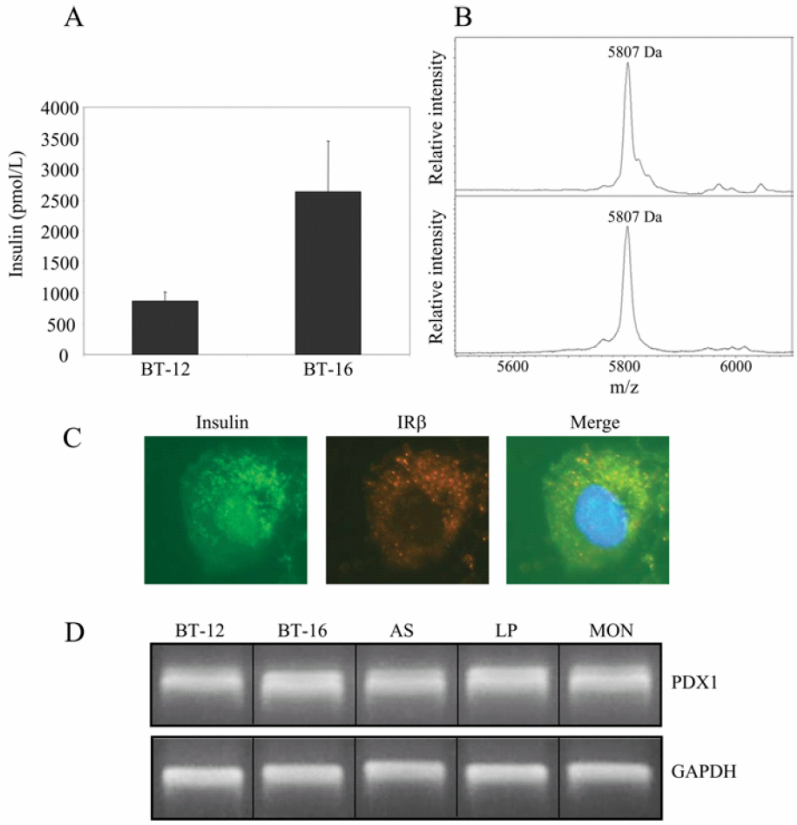

Figure 2 Human AT/RT cells secrete insulin under serum-free conditions

(**A**) Serum-free supernatants from AT/RT cells were analysed after 5 days in culture by ELISA to detect insulin production. Results are the means with S.D. from a representative experiment (out of three) performed in triplicate. (**B**) Serum-free supernatants from BT-16 cells (upper panel) or purified recombinant human insulin (lower panel) were analysed by MS. (**C**) Expression of insulin (left) and the IRβ (centre) in BT-16 cells was investigated by immunofluorescence ($\times$ 100 magnification). The detection was performed using secondary antibodies coupled with FITC (insulin) or Cy3 (IRβ). The right panel shows the merged pictures and the cell nucleus stained with DAPI (4′,6-diamidino-2-phenylindole). (**D**) The expression of PDX-1 was detected in MRT and AT/RT cells using RT–PCR. GAPDH expression was analysed in parallel as a loading control.

were performed in 96-well plates (MTS assays) or 6-well plates (Western-blot analysis), using the amounts of DNA and Lipofectamine™ 2000 recommended by the manufacturer's protocol. Opti-MEM I medium (Invitrogen) was used for the transfection and replaced by growth medium after 24 h.

The siRNAs (small interfering RNAs) targeting the IRβ and IGFIRβ and non-targeting control siRNA were purchased from Dharmacon. The siRNAs were transfected into BT-12 or BT-16 cells using Lipofectamine™ 2000 (Invitrogen). The transfections were performed in 96-well plates (MTS assays) or 6-well plates (Western-blot analysis), using the amounts of siRNA and Lipofectamine™ 2000 recommended by the manufacturer's protocol. Opti-MEM I medium (Invitrogen) was used for the transfection and replaced by growth medium after 24 h. Cell responses were assessed 72 h post-transfection.

For stable expression BT-16 cells (in 10 cm dishes) were transfected with an HA-tagged INI1 construct in pcDNA3 (Invitrogen) or with empty vector using Lipofectamine™ (Invitrogen). The amounts of DNA and Lipofectamine™ used were as recommended by the manufacturer's protocol. After 48 h, the cells were diluted 1:10 in growth medium containing G418 (1.0 mg/ml). A population of resistant cells was expanded and analysed after selection. Western-blot analysis was used to confirm expression of HA-tagged INI1.

RESULTS

Expression of components of the IR and IGFIR signalling pathways in AT/RT cells

We characterized a panel of established human AT/RT cell lines (BT-12 and BT-16) [32,41], MRT lines (AS, LP, MON and STM) [9] and medulloblastoma (DAOY) for expression of RTKs (receptor tyrosine kinases) to study further their involvement in tumour cell proliferation and survival. Lysates from normal adult brain, foetal brain and foetal cerebellum were analysed in parallel as controls. Western-blot analysis revealed that MRT and AT/RT cell lines overexpressed the IR and IGFIR, as compared with the normal brain samples (Figure 1A). Expression of the EGFR [EGF (epidermal growth factor) receptor], ErbB2 and c-Kit were also detected in the panel of cell lines (Figure 1A and results not shown). In contrast, the PDGFR (platelet-derived growth factor receptor) was not detected in the cell lines under study (results not shown). The expression of components of the PI3K/Akt signalling pathway in the panel of MRT and AT/RT cell lines was then

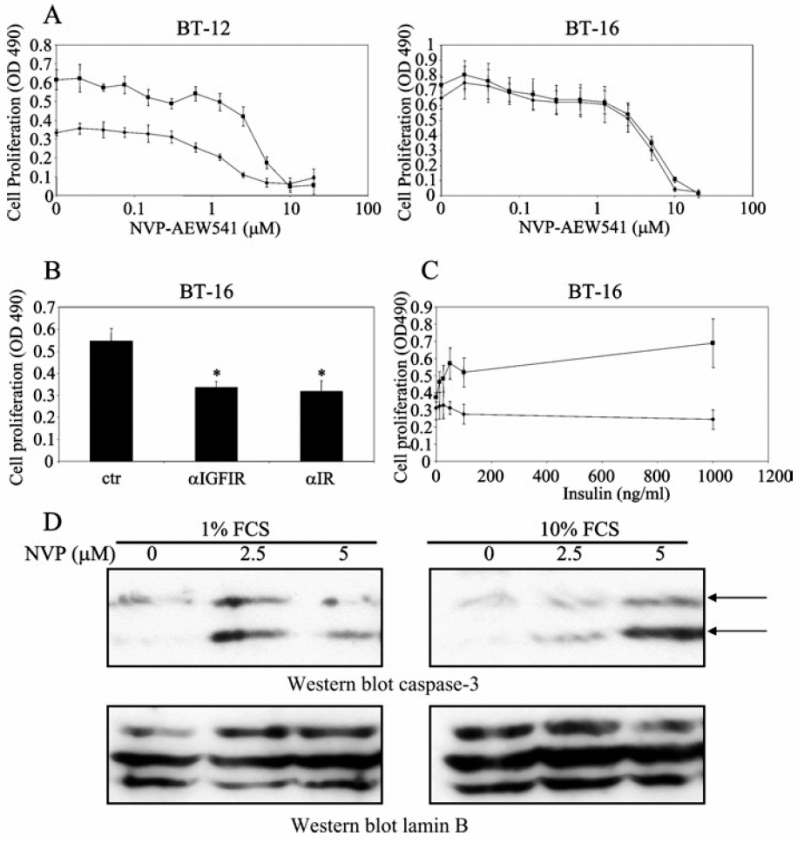

Figure 3 A pharmacological inhibitor or a neutralizing antibody targeting the IR impairs AT/RT cell proliferation

(**A**) AT/RT cells were incubated in the presence of increasing concentrations of the inhibitor NVP-AEW541 in a medium containing 10% (squares) or 0.5% (diamonds) FCS. Cell proliferation was determined after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed with eight repetitions. (**B**) BT-16 cells were incubated in serum-free medium in the presence of neutralizing antibodies targeting either the IGFIR or the IR (10 μg/ml) and cell proliferation measured after 72 h. Results are the means with S.D. from three experiments performed in quadruplicate (*, $P < 0.05$ by ANOVA test). (**C**) BT-16 cells were incubated with increasing concentrations of insulin in serum-free medium in the presence of LY294002 (10 μM, diamonds) or vehicle (squares). Cell proliferation was measured after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed with eight repetitions. (**D**) BT-12 cells were incubated for 20 h in the presence of increasing concentrations of the inhibitor NVP-AEW541 and the induction of apoptosis assessed by Western blot for the active fragments of caspase 3 (20 and 17 kDa, arrows). The blots were reprobed for lamin B as a loading control.

investigated. All cell lines expressed the PI3K regulatory subunit p85α and the p110α and p110β catalytic subunits (Figure 1A). In addition, both AT/RT cell lines overexpressed the p110δ isoform (Figure 1A). MRT and AT/RT cell lines displayed comparable levels of IRS-1 (IR substrate-1), mTOR (mammalian target of rapamycin) and ERK1/2 (Figure 1A and results not shown). The AT/RT and MRT cell lines did not express INI1, in contrast with DAOY medulloblastoma cells, as expected (Figure 1B). Indeed, AT/RT and MRT cell lines and tumours harbour inactivating biallelic alterations in the *hSNF5/INI1* gene, leading to loss of expression of the INI1 protein [1,9]. In contrast, these mutations are not found in medulloblastoma [43].

Human AT/RT secrete insulin in an autocrine manner

We next investigated whether AT/RT cells produced growth factors in an autocrine fashion. An ELISA revealed that both BT-12 and BT-16 cell lines secreted insulin into the growth medium, when cultivated under serum-free conditions (Figure 2A). The BT-16 cell line appeared to produce higher amounts of insulin than BT-12 (2628 pmol/l versus 867 pmol/l). IGF-I or IGF-II secretion could not be detected in either BT-12 or BT-16 cells grown in serum-free medium (results not shown). However, an RT–PCR analysis revealed that IGF-I mRNA was expressed in BT-16 cells, while IGF-II expression was detectable in BT-12 cells (see Supplementary Figure 1 at http://www.BiochemJ.org/bj/406/bj4060057add.htm). To confirm that the growth factor secreted by BT-16 cells corresponded to human insulin, supernatants of BT-16 cells were analysed by MS. MS analysis revealed the presence of a protein of 5807 Da, corresponding to human insulin in serum-free supernatants obtained from BT-16 cells (Figure 2B). Immunofluorescence analysis revealed that individual BT-16 cells co-expressed insulin and the IR, indicating the presence of an autocrine signalling loop in these cells (Figure 2C). We next performed RT–PCR analysis to investigate the expression of the transcription factor PDX-1 in the panel of cell lines. PDX-1 is a master regulator of pancreas development and β-cell function, which participates in the transcription of several genes, including *insulin* [44]. This analysis revealed strong expression of PDX-1 mRNA in the BT-12, BT-16 and MRT cell lines, confirming their

insulin-producing phenotype (Figure 2D). We next investigated whether other human cancer cell lines also secrete insulin under similar culture conditions. However, an analysis of a panel of human medulloblastoma and neuroblastoma cell lines failed to document insulin secretion under serum-free culture conditions (results not shown), indicating that the response is selective for CNS AT/RT cells.

Inhibition of AT/RT cell proliferation by targeting the IR

The specific IGFIR/IR kinase inhibitor NVP-AEW541 [30] inhibited proliferation of BT-12 and BT-16 cell lines (Figure 3A), in a dose-dependent manner with IC_{50} values of 2 μM in BT-12 and 5 μM in BT-16 cells (Figure 3A). In BT-12 cells, NVP-AEW541 was effective at slightly lower concentrations, when cells were incubated in medium containing low serum, as compared with high serum (Figure 3A). In contrast, no differences in sensitivities were observed in the BT-16 cell line (Figure 3A). To confirm these observations, cell proliferation of BT-12 or BT-16 cells was assessed in the presence of neutralizing antibodies specific either for the IGFIR or for the IR. Proliferation of BT-16 cells in serum-free medium was significantly inhibited by the anti-IR neutralizing antibody (Figure 3B). A similar effect was observed with the anti-IGFIR neutralizing antibody (Figure 3B). The anti-IR neutralizing antibody also significantly inhibited proliferation of BT-12 and BT-16 cells in serum-containing medium (results not shown). Increasing concentrations of insulin stimulated proliferation of BT-16 cells in serum-free medium (Figure 3C) and the response was completely inhibited by a pharmacological PI3K inhibitor (LY294002), indicating that PI3K is essential for insulin-stimulated cellular responses in AT/RT cells. The maximal increase in cell proliferation was observed at an insulin concentration of 50 ng/ml (8.6 nM) and concentrations up to 1000 ng/ml had no significantly higher effect (Figure 3C). Inhibiting IGFIR/IR function with NVP-AEW541 also induced apoptosis in AT/RT cells, as assessed by caspase 3 activation (Figure 3D), indicating that the autocrine signalling loop involving the IR contributes to cell survival.

An RNAi approach was used to confirm the critical role of the IR and IGFIR in supporting AT/RT cell growth and survival. Transfection of BT-12 and BT-16 cells with siRNA targeting either the IRβ or the IGFIRβ resulted in a significant decrease in cell viability (Figures 4A and 4B and results not shown). To verify the specificity of the effect of the siRNA targeting the IRβ, protein down-regulation was assessed by Western-blot analysis in BT-12 and BT-16 cells (Figure 4C and results not shown).

Activation of the PI3K/Akt signalling pathway by insulin in AT/RT cells

The ability of polypeptide growth factors to activate PI3K/Akt, ERK1/2 and ribosomal protein S6K was then investigated in BT-12 and BT-16 cells. Insulin most potently activated Akt, as compared with EGF and SCF (stem cell factor), whereas activation of ERK1/2 was comparable between all growth factors (Figure 5A). The IGFIR/IR kinase inhibitor NVP-AEW541 (2.5 μM) completely inhibited IGF-I- and insulin-activated responses (Figure 5B). Autophosphorylation of the IR was also detected in insulin-stimulated AT/RT cells, confirming the specificity of the responses observed (Figure 5C).

Our data so far had revealed activation of the PI3K/Akt, S6K and ERK1/2 pathways in AT/RT cells stimulated by polypeptide growth factors. Therefore we next investigated the contributions of these pathways to proliferation in BT-12 and BT-16 cells.

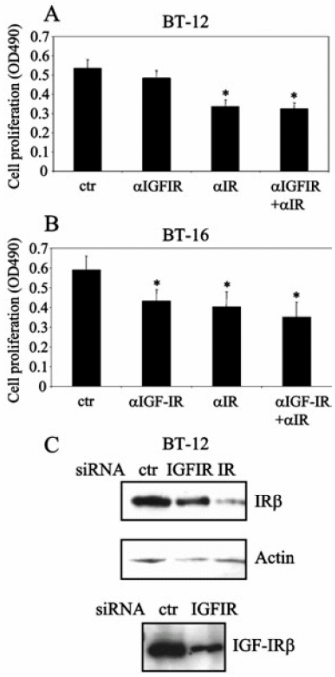

Figure 4 Down-regulation of IR by siRNA treatment impairs AT/RT cell proliferation

(**A**, **B**) BT-12 or BT-16 AT/RT cells were transiently transfected with non-targeting siRNA (ctr), or siRNA targeting the IGFIR or the IR, alone or in combination, where indicated. Cell proliferation was measured after 72 h. Results are the means with S.D. from three experiments performed in quadruplicate (*, $P < 0.05$ by ANOVA test). (**C**) BT-12 AT/RT cells were transiently transfected with siRNAs as indicated and expression of the IRβ and IGFIRβ was analysed by Western blot after 72 h.

Cell proliferation was assessed in the presence of the PI3K inhibitor LY294002, rapamycin, the MEK [MAPK (mitogen-activated protein kinase)/ERK kinase] inhibitor PD98059, or a pharmacological Akt/PKB inhibitor. AT/RT cell proliferation was significantly impaired by LY294002 or rapamycin, in both BT-12 and BT-16 cells grown in low (1%) or high (10%) serum (Figure 5D). The Akt inhibitor completely inhibited proliferation of the BT-12 cell line in low, but not high serum, while having a lesser effect in the BT-16 cell line (Figure 5D). To confirm the involvement of PI3K/Akt signalling in AT/RT cell proliferation, a dominant-negative catalytically inactive Akt (AktDN) construct was transiently transfected into BT-12 and BT-16 cells. Western-blot analysis confirmed expression of the construct in AT/RT cells (results not shown). The AktDN construct significantly inhibited proliferation in both cell lines (Figure 5E). In contrast, transfection of an activated Akt mutant (myrAktCA) had no significant effect on AT/RT cell proliferation.

The class I_A PI3K isoform p110α controls AT/RT cell proliferation and insulin-stimulated Akt activation

To confirm the involvement of PI3K signalling in AT/RT cell responses to insulin, a panel of isoform-specific class I_A PI3K inhibitors was tested in AT/RT cells. The pharmacological p110α inhibitor YM024 [45] inhibited proliferation of AT/RT cells in a dose-dependent manner with an IC_{50} of 1.5 μM (Figure 6A). In contrast, the p110β inhibitor TGX-221 [46] and the p110δ

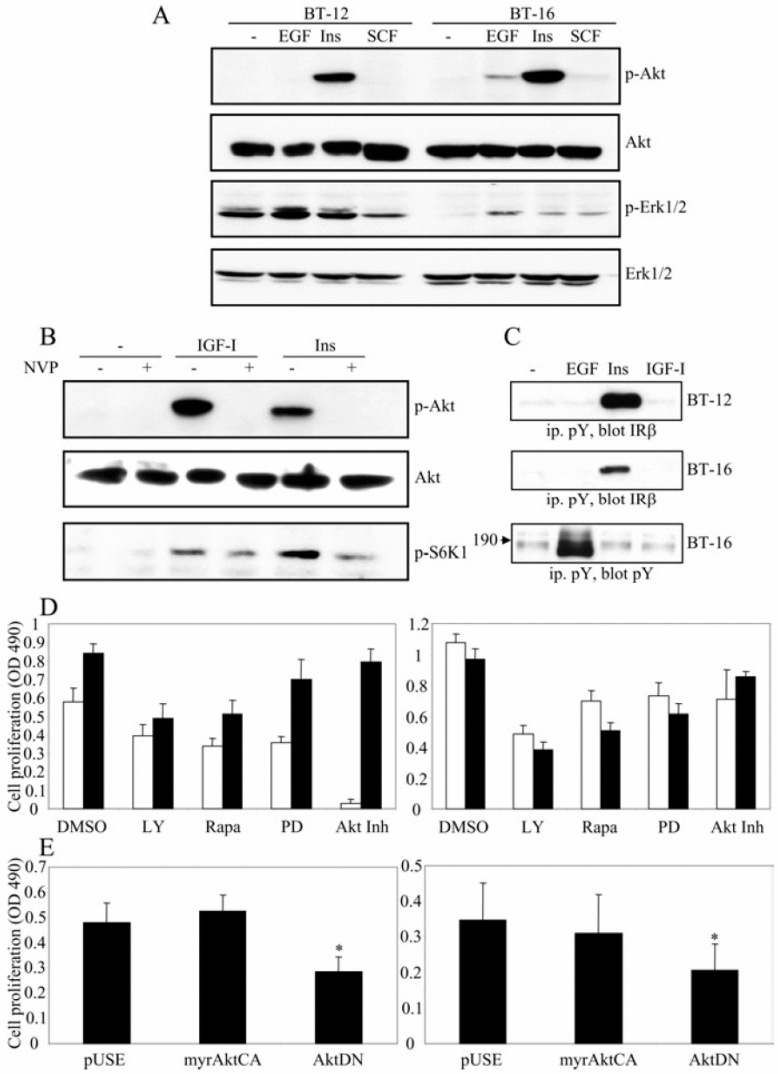

Figure 5 Insulin activates the PI3K/Akt pathway in AT/RT cells

(**A**) Serum-starved AT/RT cells were stimulated with growth factors where indicated (40 ng/ml EGF; 50 ng/ml Ins; 20 ng/ml SCF) for 10 min, and activation of Akt and ERK was determined by Western blot with phospho-specific antibodies. Abbreviations: p-Akt, Ser^{473}-phosphorylated Akt; p-ERK, Thr^{202}/Tyr^{204}-phosphorylated ERK. (**B**) Serum-starved BT-16 cells were pretreated with NVP-AEW541 (2.5 μM), stimulated with the growth factors indicated (50 ng/ml) for 10 min and activation of Akt and S6K1 was determined as above. Abbreviation: p-S6K1, Thr^{389}-phosphorylated S6K. (**C**) Serum-starved AT/RT cells were stimulated with the growth factors indicated (10 min) and anti-phosphotyrosine (pY) immunoprecipitates were analysed by Western blots for the relevant receptors. (**D**) BT-12 (left panel) or BT-16 (right panel) cells were incubated in a medium containing 10 % (closed bars) or 0.5 % (open bars) FCS in the presence of the inhibitors indicated [LY294002 10 μM (LY); rapamycin 20 ng/ml (Rapa); PD98059 25 μM (PD); Akt inhibitor 20 μM (Akt Inh)], or vehicle (DMSO). Cell proliferation was determined after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed with eight repetitions. (**E**) BT-12 (left panel) or BT-16 (right panel) cells were transiently transfected with empty vector, or constructs encoding activated (myrAktCA) or dominant-negative Akt (AktDN). Cell proliferation was determined after 72 h. Results are the means with S.D. from three experiments performed in quadruplicate (*, $P < 0.05$ by ANOVA test).

inhibitor IC87114 [47] had a less pronounced effect on AT/RT cell proliferation (Figure 6A). The pharmacological p110α inhibitor YM024 also inhibited insulin-stimulated Akt activation, whereas TGX-221 and IC87114 did not impair the response (Figure 6B and results not shown). shRNA constructs targeting the catalytic subunits of class I_A PI3Ks were then transiently transfected into BT-16 cells to investigate their impact on cell proliferation. Western-blot analysis confirmed down-regulation of the expression of the target genes by the relevant shRNA construct (Figure 6D). The shRNA constructs targeting *PIK3CA* (encoding p110α) strongly (> 80 %) inhibited BT-16 cell proliferation (Figure 6C). In contrast, RNAi targeting of *PIK3CB* resulted in no significant effect on AT/RT cell proliferation (Figure 6C). Moreover, insulin-stimulated Akt activation was abrogated by transfection of BT-16 cells with the shRNA construct targeting *PIK3CA* (results not shown). Thus the p110α isoform appears to play a crucial role in controlling AT/RT cell proliferation and insulin-stimulated Akt activation.

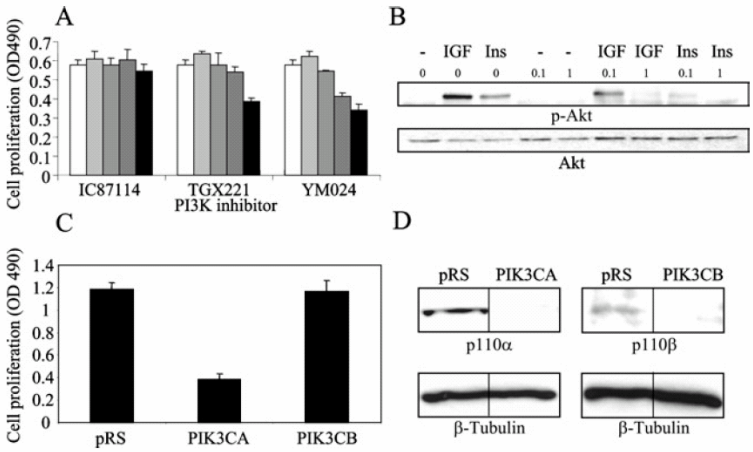

Figure 6 The PI3K p110α isoform controls AT/RT cell proliferation and insulin signalling

(**A**) BT-16 cells were incubated with increasing concentrations of isoform-specific PI3K inhibitors [0.01 μM (light grey bars), 0.1 μM (medium grey bars), 1 μM (dark grey bars), 10 μM (closed bars) or vehicle (open bars)]. Cell proliferation was determined after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed in quadruplicate. (**B**) Serum-starved BT-16 cells were pre-incubated with the p110α inhibitor YM024 (micromolar concentrations) and stimulated with growth factors (50 ng/ml insulin; 50 ng/ml IGF-I) where indicated for 10 min. Akt activation was determined by Western blot with phospho-specific antibodies. Abbreviation: p-Akt, Ser^{473}-phosphorylated Akt. (**C**, **D**) BT-16 cells were transiently transfected with shRNA constructs targeting PI3K isoforms (PIK3CA: p110α; PIK3CB: p110β) or empty vector (pRS). (**C**) Cell proliferation was assessed after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed with eight repetitions. (**D**) Protein down-regulation was determined by Western-blot analysis.

Ectopic expression of INI1 does not affect the IR signalling pathway in AT/RT cells

Since AT/RT cells frequently display mutations in the *INI1* gene, we investigated the impact of ectopic re-expression of the protein in BT-16 cells. BT-16 stably transfected with an INI1 expression vector displayed reduced proliferation under either low- or high-serum culture conditions (Figure 7A). However, BT-16 transfected with INI1 still displayed detectable insulin mRNA expression (Figure 7C). Moreover, the expression levels of the IR and IGFIR were not affected, as assessed by Western-blot analysis (Figure 7D). These results indicate that the establishment of the autocrine loop involving insulin and its receptor is independent of *INI1* loss in AT/RT cells.

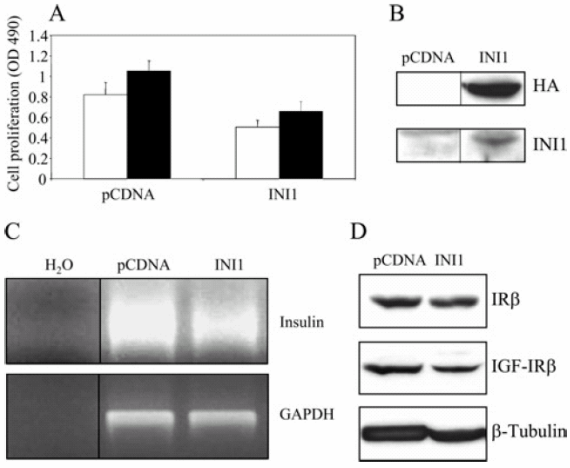

Figure 7 Ectopic expression of *INI1* reduces the proliferation of AT/RT cells, without effect on IR expression

(**A**) BT-16 cells were stably transfected with empty vector (pcDNA) or HA-tagged INI1. Cell proliferation in a medium containing 0.5% (open bars) or 10% (closed bars) FCS was determined after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed with eight repetitions. (**B**) Expression of HA-tagged INI1 in the transfected BT-16 cells was verified by Western-blot analysis. (**C**) Insulin mRNA expression in BT-16 cells transfected with empty vector (pcDNA) or HA-tagged INI1 was determined by RT–PCR. GAPDH was analysed in parallel as a control. (**D**) The expression of the IRβ and the IGFIRβ in BT-16 cells transfected with empty vector (pcDNA) or HA-tagged INI1 was determined by Western blot.

DISCUSSION

Molecular abnormalities linked with human AT/RT include germline and somatic mutations in the *INI1* gene that encodes a chromatin remodelling factor and has been suggested to function as a tumour suppressor [7–9]. A recent report has also documented epigenetic repression of the *RASSF1* (Ras association domain family 1) gene in AT/RT cell lines and primary tumours [41]. RTK signalling has not yet been studied in detail in AT/RT cells. Expression of the IGFIR and IGF-II was reported in a limited number of cases of AT/RT tumours [25,48]. Targeting the IGFIR with antisense oligonucleotides resulted in increased apoptosis and sensitivity to the chemotherapeutic agents cisplatin and doxorubicin in AT/RT cells [32]. In the case of the MRTs, the EGFR inhibitor gefitinib was reported to have anti-tumour effects *in vitro* and *in vivo* [49]. Moreover, the PI3K/Akt pathway was reported to be involved in the resistance of MRT cells to chemotherapy and radiotherapy [50].

We have characterized a panel of human MRT and AT/RT cell lines for expression of RTKs and their downstream signalling mediators, in order to study the potential of targeting these molecules to inhibit cell proliferation and survival. The IR and IGFIR were found overexpressed in both AT/RT and MRT cell lines, as compared with normal brain tissue. Moreover and surprisingly, AT/RT cell lines secreted insulin when grown under serum-free conditions, indicating autocrine signalling events. To our knowledge these AT/RT cell lines are the first example of human CNS tumour cell lines secreting insulin. Moreover, the

only other examples of insulin secretion by cells other than pancreatic β-cells are sperm cells [51]. The concentration of insulin measured in supernatants from BT-16 cells was 2.5 nM, which is in the biological range. Maximal biological responsiveness to insulin tends to occur at concentrations in the range 10–20 nM [52–54] and insulin had maximal effects on BT-16 cell proliferation at a concentration of 8.6 nM. It is conceivable that AT/RT cells also produce other growth factors in an autocrine fashion. Indeed, IGF-I and IGF-II were detected at the mRNA level in BT-16 and BT-12 cells respectively, as recently reported [32]. We were unable to detect free IGF-I and IGF-II in supernatants from AT/RT cells by ELISA, but this could be due to the fact that IGFs were bound to IGF-binding proteins. Autocrine signalling by IGF-I via the IGFIR in BT-16 cells would explain the inhibitory effects of IGFIR-neutralizing antibodies or siRNA on the proliferation of these cells.

Insulin activated PI3K/Akt, S6K and ERK signalling, whereas other growth factors such as EGF or SCF selectively activated some, but not all of the pathways. Targeting the IGFIR kinase activity with NVP-AEW541 resulted in inhibition of AT/RT proliferation, although at higher IC_{50} values than reported in other tumour cells [30]. These increased IC_{50} values possibly reflect co-expression of the IR and IGFIR in AT/RT cell lines, since the IC_{50} of NVP-AEW541 for the IR was reported to be 2.3 μM in cells [30,55]. This model was supported by the observations that neutralizing antibodies or siRNA targeting the IRβ inhibited the proliferation of AT/RT cells. Consequently, our results demonstrate that insulin secreted by AT/RT cells in an autocrine fashion activates the IR and thus contributes to AT/RT cell proliferation and survival. This mechanism of IR activation appears to be different from the previously reported activation loop involving IGF-II and the IR isoform A in human cancer cells [56–58]. The molecular mechanisms underlying the establishment of the autocrine insulin signalling loop in AT/RT cells are currently unclear. Loss of *hSNF5/INI1* did not appear to be a major cause of the expression of insulin and the IR by AT/RT cells, since transfection of INI1 did not substantially alter the expression of these molecules. Intriguingly, the pancreatic transcription factor PDX-1 was detected in AT/RT and MRT cells, indicating that aberrant expression of transcription factors may drive these cancer cells towards an insulin-producing phenotype. A recent survey of mutations in human tumour genomes has revealed frequent mutations in transcription factors, emphasizing the contribution of abnormalities in transcription to the development of human cancer [59].

To investigate whether targeting downstream signalling mediators of the IR could provide a means of inhibiting proliferation of AT/RT cells, we used a combination of pharmacological and RNAi approaches. This revealed that the PI3K/Akt/mTOR pathway was crucial in the control of AT/RT cell proliferation. Moreover, a selective role for the PI3K isoform p110α in transducing signals from the IR was uncovered in AT/RT cells, in good agreement with recent reports documenting the crucial role of p110α in insulin signalling [60,61]. Thus pharmacological inhibitors targeting the PI3K p110α isoform may have antiproliferative potential in human cancer cells where this isoform is activated by the IR.

Insulin has various biological effects in a broad range of tissues, and its functions in the brain include neuronal survival and regulation of energy homoeostasis [62]. Although a large body of evidence exists implicating IGF-I and IGF-II as autocrine growth factors in a broad range of human malignancies [24,27], such a function has not yet been described for insulin in CNS tumours. In the light of the novel function for insulin as an autocrine growth factor for CNS AT/RT cells, further studies are warranted to uncover its potential role in other human cancers.

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3.2.4 Targeting PI3KC2β impairs proliferation and survival in acute myeloid leukemia, brain tumours and neuroendocrine tumours *(Submitted to the British Journal of Cancer)*

Targeting PI3KC2β impairs proliferation and survival in acute leukemia, brain tumours and neuroendocrine tumours

Running Title: PI3KC2β in human cancers

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ABSTRACT

Eight mammalian phosphoinositide 3-kinase (PI3K) isoforms exist which are subdivided into three classes. While much attention has been given to the class I isoforms, little is known about the functions of class II PI3Ks in human cancer. The expression pattern and functions of the PI3KC2β isoform were investigated in a panel of tumour samples and cell lines. Over-expression of PI3KC2β was found in subsets of tumours and cell lines from acute myeloid leukemia (AML), glioblastoma multiforme (GBM), medulloblastoma (MB), neuroblastoma (NB), and small cell lung cancer (SCLC). Specific pharmacological inhibitors of PI3KC2β or small interfering RNA (siRNA) impaired proliferation of a panel of cell lines and primary cultures from AML, brain tumours and neuroendocrine tumours. Inhibition of PI3KC2β also induced apoptosis in AML and GBM cell lines and sensitised the cells to chemotherapeutic agents. Furthermore, PI3KC2β inhibition impaired the phosphorylation of downstream signalling mediators in AML. Together, these data show that PI3KC2β contributes to proliferation and survival in AML, brain tumours and neuroendocrine tumours and may represent a novel target in these malignancies.

Keywords: PI3KC2β, pharmacological inhibition, cell proliferation, migration

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INTRODUCTION

Phosphoinositide 3-kinases (PI3Ks) play an essential role in the signal transduction events initiated by the binding of extracellular signals to their cell surface receptors (Katso et al., 2001; Vanhaesebroeck et al., 2001). The cellular responses controlled by PI3Ks are extremely diverse, including mitogenesis and proliferation, protection from apoptosis and cell motility (Katso et al., 2001; Vanhaesebroeck et al., 2001). There are eight known PI3Ks in humans, which have been subdivided into three classes, based on structural homology and *in vitro* substrate specificity (Vanhaesebroeck et al., 1997a; Vanhaesebroeck & Waterfield, 1999). Class I_A comprises three highly homologous isoforms, p110α (Hiles et al., 1992), p110β (Hu et al., 1993) and p110δ (Chantry et al., 1997; Vanhaesebroeck et al., 1997b), which exist as a heterodimeric complex with a regulatory subunit containing two Src homology-2 (SH2) domains, mediating enzyme association with phosphotyrosine residues in the cytoplasmic domains of activated polypeptide growth factor receptors (Inukai et al., 1996; Otsu et al., 1991; Pons et al., 1995). All class I PI3Ks function as PtdIns(4,5)P_2 3-kinase *in vivo*, upon activation by receptor tyrosine kinases or serpentine receptors (Hawkins et al., 1992; Stephens et al., 1991). PtdIns(3,4,5)P_3 serves as a docking site for the serine/threonine protein kinase phosphoinositide-dependent protein kinase-1 (PDK-1) which is activated upon binding (Alessi et al., 1997). Several protein kinases have been identified as downstream targets of PDK-1, such as the serine/threonine protein kinase B (PKB) /Akt, which is a key regulator of cell survival, ribosomal protein S6 kinase (S6K), which stimulates protein synthesis and cell growth, glycogen synthase kinase-3 (GSK-3), a key regulator of glycogen synthesis, and a subset of protein kinase C (PKC) isoforms (Le Good et al., 1998; Vanhaesebroeck & Alessi, 2000).

Class II PI3Ks comprise the Drosophila PI3K_68D/Cpk (MacDougall et al., 1995; Molz et al., 1996), mouse Cpk-m (Molz et al., 1996), and human PI3KC2α (Domin et al., 1997), PI3KC2β (Arcaro et al., 1998) and PI3KC2γ (Misawa et al., 1998; Ono et al., 1998). The hallmarks of class II family members are a substrate specificity restricted to PtdIns and PtdIns(4)P *in vitro*, and a conserved C-terminal C2 domain, involved in phospholipid binding. Recent studies have started to investigate the regulation and functions of class II PI3Ks *in vivo*. Indeed, several reports have shown that class II PI3Ks are downstream targets of activated receptor tyrosine kinases (RTKs), such as the epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), c-Kit and insulin receptor (IR) (Arcaro et al., 2002; Arcaro et al., 2000; Brown et al., 1999; Katso et al., 2006). Studies in *Drosophila melanogaster* have also revealed a role for the class II PI3K_68D in cell differentiation downstream of the EGFR (MacDougall et al., 2004). Furthermore, recent reports have documented a role for PI3KC2β in cell migration in mammalian cells via activation of Rho family GTPases and in human cancer cells (Domin et al., 2005; Katso et al., 2006; Maffucci et al., 2005). Studies by others have described a role for PI3KC2α in Rho activation and contraction in vascular smooth muscle cells (Wang et al., 2006). Analysis of a conditional knock-out mouse of *PIK3C2B*, however, revealed no obvious phenotype despite a fairly ubiquitous deletion of *PIK3C2B* (Harada et al., 2005).

The importance of PI3K signalling in cancer is highlighted by the fact that mutations in the tumour suppressor gene phosphatase and tensin homologue (*PTEN*) occur frequently in human tumors. PTEN is a phosphatase that antagonises the action of PI3K by de-phosphorylating the D-3 position of

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polyphosphoinositides (Cantley & Neel, 1999; Sansal & Sellers, 2004). Moreover, recent reports have described activating mutations in the *PIK3CA* gene encoding the catalytic p110α isoform of PI3K in a variety of human cancers including breast, colon and ovarian cancers (Broderick et al., 2004; Samuels et al., 2004; Weir et al., 2004).

In the present report we have evaluated the expression of the class II PI3KC2β isoform in a panel of primary human tumours and cell lines. Furthermore, we have used isoform-specific pharmacological inhibitors and RNAi to inhibit this enzyme in human cancer cell lines. We show for the first time that PI3KC2β is over-expressed in acute myeloid leukemia, brain tumours and neuroendocrine tumours and that inhibiting this class II PI3K decreases proliferation and survival in cell lines from these cancers.

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MATERIALS AND METHODS

Reagents and antibodies

Antibodies and reagents were purchased from the following companies: The PI3KC2β antibody was described in (Arcaro et al., 1998). Caspase-3, PARP (Santa Cruz Biotechnology, Santa Cruz, CA, USA); activated Akt/PKB (Ser473), activated JNK (Thr183/Tyr185), activated S6 protein (Ser235/236) (Cell Signalling Technology, Danvers, MA, USA); β-actin, β-tubulin (Sigma-Aldrich, St Louis, MO, USA); siGENOME™ siRNA (Dharmacon, Lafayette, CO, USA);. (PIramed, Berkshire, UK); Etoposide (Calbiochem, La Jolla, CA, USA); Doxorubicin (Pfizer AG, Zurich, CH). PI701 (YM185453) and PI702 (YM182832) were provided by Piramed Pharma Limited and their synthetic details will be the subject of a separate publication.

Apoptosis

For detection of apoptosis, cells were incubated for 16-24 hrs in the presence or absence of inhibitors. The cells were lysed and caspase-3 activity was measured using the CaspACE Assay System (Promega). Additionally, samples were analysed by SDS-PAGE and Western blot with anti-caspase-3 or anti-poly(ADP-ribose) polymerase (PARP) antibodies.

Cell culture

Acute myeloid leukemia, neuroblastoma, glioblastoma, medulloblastoma, small cell lung cancer and mammary epithelial cell lines were grown in RPMI or DMEM (Life Technologies/InvitrogenCarlsbad, CA, USA) with 10% (v/v) fetal calf serum (FCS) and penicillin/streptomycin/L-glutamine, and passaged every 3-5 days. DAOY, D341, D425 and D458 medulloblastoma cell lines were grown in MEM Zinc option (Richter's modification) medium supplemented with 10% FCS. For growth factor stimulations cells were incubated overnight in their growth medium with low serum (0.5-1% v/v) or Optimem medium (Life Technologies/Invitrogen) and washed with serum-free medium prior to incubation with growth factors. Heparinized peripheral blood or bone marrow samples were obtained from adult patients with AML. Blast cells were isolated as described previously (Doepfner et al., 2007). Type II human lung pneumocytes (Pardo et al., 2001) were maintained in DCCM-1 medium supplemented with 10% new born calf serum.

Cell proliferation

Cell lines (5 x 10^3 cells/well) were seeded in 96-well plates and grown for 72h in serum (10%)-containing medium in the presence or absence of inhibitors. The number of viable cells was analysed by means of an MTS assay using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Data are mean with SD from 8 repetitions.

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Dissociation of brain tumours

Human brain tumours were removed from 4 patients who underwent surgery for tumour resection at the University Hospital Zurich. The procedures were conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Canton Zurich. Following removal, tumour tissue was immediately placed in a petri dish, minced mechanically and digested enzymatically with collagenase D and DNase I (Roche Applied Science, Rotkreuz, Switzerland) for 1 hour at 37°C while being stirred with a magnetic bar. The dissociated cells were then sequentially filtered through 100 and 70µm cell strainers (BD Falcon, BD Biosciences, Basel, Switzerland) to remove any tissue debris. Erythrocytes were removed by resuspending and incubating the cells in ice-cold ACK buffer (17 mM Tris–HCl [pH 7.2] containing 144 mM NH_4Cl) for 10 minutes on ice. The cells were washed in PBS and plated in DMEM (Life Technologies/Invitrogen) supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS) and gentamycin (20mg/ml) and passaged every 3-5 days by trypsinization.

DNA Microarray

Total RNA was extracted from 60 medulloblastoma samples (MB) and 3 cell lines (D283, D341, and DAOY) using the Trizol reagent (Invitrogen). After DNAse treatment and RNA purification (RNeasy Micro kit, Qiagen), gene expression profiles were obtained on the Affymetrix HG-U133 Plus 2.0 array that contains more than 54000 probe sets for transcripts and variants. Expression data for 9 normal cerebellums analyzed on the same Affymetrix array version were obtained from Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). Gene expression data for the samples were normalized using the GCRMA procedure. PI3KC2β expression levels for the samples are presented (unlogged data).

Isolation of RNA from tumour samples and RT-PCR

For primary neuroblastoma samples ethical approval to use residual tissue was obtained. RNAlater-preserved tumour tissue was available in the Swiss Pediatric Oncology Group tumour bank. All diagnoses were confirmed by histological assessment of the tumour specimen obtained at surgery. Neuroblastoma tissue was disrupted with a sterile disposable tissue grinder (Sage Products, Cary, IL, USA) and homogenised in guanidinium isothiocyanate-containing buffer. Total RNA of cell lines or tumour tissue was isolated using the RNeasy kit (Qiagen, Santa Cruz, CA, USA) according to the manufacturer's protocol. Total RNA (3 µg) from each tumour sample was converted into cDNA using the SuperScript™ First-Strand Synthesis System for PCR according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). mRNA expression of PI3KC2β and 18S (internal control gene) was measured in tumour samples and cell lines by TaqMan® Assay-on-Demand™ Gene Expression products (Applied Biosystems, Foster City, CA, USA). The following primers were used (gene - assay ID): PI3KC2beta - Hm00153248_m1; eukaryotic 18S rRNA - Hs99999901_s1. Three replicates were run for each sample in a 96-well format plate. Gene expression assays consisted of a FAM™ dye-labelled TaqMan® MGB probe and two PCR primers. The thermal cycling

conditions consisted of an initial denaturation step at 95°C for 10 min and a 50-cycle countdown at 95°C for 15 s and 60°C for 1 min. Each sample was normalised on the basis of its 18S rRNA content. Relative mRNA expression levels were calculated using the comparative threshold cycle (CT) method (Giulietti et al 2001).

PI3K Assays

Recombinant human PI3KC2β was expressed as a glutathione *S*-transferase (GST)-fusion protein in SF9 insect cells and purified as described previously (Arcaro et al., 1998). Recombinant class I PI3K isoforms were expressed and purified likewise. PI3K activity of the different PI3K isoforms was assayed essentially as described (Hayakawa et al., 2007).

SDS-PAGE and Western blot analysis

Cellular lysates were prepared as previously described (Guerreiro et al., 2006) separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Biosciences), and immunoblotted with various antibodies according to the manufacturer's protocol. Chemiluminescence was used for visualization using the enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Biosciences) according to the manufacturer's protocol.

Transient expression in AML cells

AML cells were transfected with small interfering RNA (siRNA) targeting PI3KC2β using the Amaxa Nucleofector system (Amaxa biosystems, Gaithersburg, MD, USA) according to the manufacturer's protocol. Cell Line Nucleofector Kit V was used and program V-001 applied. After 48h cells were lysed in cell lysis buffer in order to visualize protein expression by SDS-PAGE and Western blotting. Besides, cells were analysed for cell proliferation and apoptosis by MTS assay and Caspase-3 measurement 72h after transfection.

Wound healing assay

EpH4 murine epithelial cells stably transfected with the Ha-*Ras* oncogene and induced by TGF-β1 to undergo epitheliomesenchymal transition (EMT) *in vivo* to establish FibRas cells were previously described in (Maschler et al., 2005). For wound healing assays, cells were plated in 12-well culture plates in complete medium and grown to confluency. A wound was created by scraping cells with a 200µl tip. The migration rate was monitored for eight hours by phase contrast microscopy (Leica DM IRBE Inverse, Widefield) either in the presence or absence of inhibitors.

RESULTS

Expression of PI3KC2β in tumour samples and cell lines

Previous reports had documented increased expression of PI3KC2β in leukemia, glioblastoma and lung cancer cell lines and tumours (Arcaro et al., 2002; Knobbe & Reifenberger, 2003; Qian et al., 2002). Therefore, the expression of PI3KC2β was investigated in a panel of tumours and cell lines from AML, neuroendocrine tumours (SCLC and NB) and brain tumours (MB and GBM). In AML, PI3KC2β was highly expressed in a subset of AML blasts and cell lines (Figure 1A, left panel). Interestingly, PI3KC2β expression was much lower in non-leukemic bone marrow cells and immortalised B cells (Figure 1A) indicating that AML blasts and cell lines over-express the enzyme. We also reanalysed cDNA microarray data from a previously published study in AML (Valk et al., 2004). PI3KC2β mRNA expression was found to be higher in certain groups of AML, depending on molecular and cytogenetic abnormalities. AML categories displaying increased expression included FLT3-ITD and EVI1 (77.37; 99.21), as compared to FLT3-TKD (41.51). Increased expression of PI3KC2β was also found in AML with the cytogenetic abnormalities -7 (99.72), when compared to +8, 11q13, t(8;21), idt(16), or NN (46.86; 39.35; 54.40; 19.80; 60.03). In contrast, there were no significant differences in expression of PI3KC2β mRNA between AML French-American-British (FAB) classes.

Protein expression analysis of SCLC cell lines revealed elevated PI3KC2β expression in 2/4 cases when compared to a normal Type II pneumocyte cell line (PN) (Figure 1B, left panel). TaqMan analysis of *PIK3C2B* expression confirmed that mRNA levels are predictive of protein expression (Figure 1B, right panel).

In neuroblastoma (NB) cell lines, Western blot analysis revealed broad expression of PI3KC2β (Figure 2A). Upon TaqMan analysis *PIK3C2B* was found to be over-expressed at the mRNA level when compared to normal human adrenal gland (5/8 samples with >2-fold expression) (Figure 2B). In primary tumours from children under the age of one year PI3KC2β showed increased expression (Figure 2C). This distribution was mirrored in data obtained from TaqMan analysis, where increased mRNA expression was found in the same patient subgroup (3/19 samples with >2-fold expression) (Figure 2D).

In glioblastoma multiforme (GBM) cell lines and *ex vivo* cultures PI3KC2β was found to be over-expressed in a subset of samples when compared to normal human brain or cerebellum (Figure 2E).

In medulloblastoma (MB) cell lines, heterogeneous PI3KC2β expression was observed (Figure 2F). Microarry analysis of a panel of primary medulloblastoma samples also showed that PI3KC2β was over-expressed in 16/60 samples compared to normal human cerebellum (Supplemental Figure 1).

Together, these data revealed subgroups of tumours and cell lines displaying PI3KC2β over-expression, in which the class II PI3K may play a role in regulating proliferation, survival or migration.

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Inhibition of cell proliferation by pharmacological PI3KC2β inhibitors or siRNA targeting PI3KC2β

To gain insight into the contribution of the class II PI3K isoform PI3KC2β in cell proliferation, two different isoform-specific pharmacological inhibitors (PI701 and PI702) were used. The specificity of these inhibitors was verified by *in vitro* PI3K assays using purified recombinant preparation of various PI3K isoforms. The IC_{50} values for specific inhibition of the enzymatic activity of PI3KC2β were 182 nM for PI701 and 652 nM for PI702 while values above 10μM were observed for the other PI3K isoforms (Table I). Both inhibitors also failed to inhibit the activity of mTOR and DNA-PK, with IC_{50} values above 100 μM (data not shown). A screen against a panel of 72 protein kinases *in vitro* confirmed that the two compounds are selective for PI3KC2β. Indeed, they only showed some inhibitory activity, at a concentration of 10 μM *in vitro*, against anaplastic lymphoma kinase (ALK), c-Raf, p38-regulated/activated kinase (PRAK) and tyrosine protein kinase receptor B (TrkB)* (*PI702 only) (data not shown).

The anti-proliferative activity of both inhibitors was investigated in a panel of 28 cell lines and primary cultures from AML, GBM, MB, NB and SCLC. As controls, non-leukemic bone marrow cells (N), two immortalised B cells lines (FIN COS, 41b MI) and an immortalised Type II pneumocyte cell line (PN) were included. Both inhibitors of PI3KC2β inhibited cell proliferation in a dose-dependent manner with IC_{50} values below 10μM (PI701: 16/30 cases; PI702: 9/25 cases) (Figure 3 and Table II). The lowest IC_{50} values were observed in SCLC cell lines, AML blasts and D341 MB cells (Table II). In contrast, the IC_{50} values observed in control cells (FIN COS, 41b MI, N, PN) were above 10 μM. In several cases, cell lines with high PI3KC2β expression were more sensitive to PI701 and PI702, as was the case for AML (U937, NB4), MB (D341), GBM (T98G) and SCLC (H-209, H-510) (Figure 1 and 2, Table II).

Investigation of the effect of PI701 on activation of downstream signalling mediators in AML cell lines showed a dose-dependent decrease in the phosphorylation of key signalling molecules (Figure 4). These include Akt, c-Jun N-terminal kinases (JNK) and ribosomal S6 protein.

Together, these data show that inhibition of PI3KC2β with selective pharmacological inhibitors impairs proliferation in a subset of human cancer cells with IC_{50} values compatible with inhibition of the enzyme. This is in line with the finding that the phosphorylation of key signalling molecules is decreased upon treatment with PI701 in AML.

To validate the results obtained with pharmacological inhibitors, small interfering RNA (siRNA) targeting PI3KC2β was tested in a AML cell line. The specific down-regulation of PI3KC2β expression by siRNA was verified by Western blot analysis (Figure 5A). Decreased expression of PI3KC2β in U937 resulted in a 40% reduction of cell proliferation and was accompanied by increased caspase-3 activity (Figure 5B). The effects of the PI3KC2β siRNA on cell proliferation were less marked in THP1 cells, which displayed lower expression of the enzyme (data not shown).

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Targeting PI3KC2β enhances the sensitivity of AML and GBM cells to chemotherapy

We next investigated whether inhibition of PI3KC2β by pharmacological inhibitors could modulate the sensitivity of AML and GBM cells to chemotherapeutic agents. In AML cell lines, combination treatment with PI701 led to markedly increased sensitivity to etoposide (Figure 6A). This effect was also observed in GBM cell lines and *ex vivo* cultures where PI701 combined with doxorubicin had a stronger effect on cell viability than the chemotherapeutic agent alone (Figure 6B and 6C). Moreover, the combination treatment enhanced the induction of apoptosis as assessed by increased caspase-3 activation and poly (ADP-ribose) polymerase (PARP) cleavage (Figure 6D and 6E).

In summary, these results highlight the importance of PI3KC2β in regulating the viability and chemoresistance of various tumour cells.

PI3KC2β contributes to cell migration in transformed epithelial cells

Previous reports have described a role for PI3KC2β in the migration of cancer cells (Katso et al., 2006; Maffucci et al., 2005). To investigate the specific contribution of PI3KC2β in the migration of tumourigenic vs. non-tumourigenic cells, murine epithelial cells sequentially transformed by oncogenic H-Ras and TGF-β1 were used (Maschler et al., 2005). To analyse the contribution of PI3KC2β to the migratory capacity of cancer cells, murine epithelial cells were treated with PI701. Western blot analysis revealed increased PI3KC2β expression in the FibRas cells, which have previously been shown to display enhanced migration and invasion, as compared to Eph4 and EpRas cells (Maschler et al., 2005) (Figure 7A). Treatment with PI701 impaired migration of FibRas cells but had no significant effect on the migration of Eph4 and EpRas cells (Figure 7B). At the concentrations used, PI701 did not significantly inhibit cell proliferation (data not shown). The finding that pharmacological inhibition of PI3KC2β impaired the migratory capacity of highly invasive cells emphasises the importance of PI3KC2β in regulating cancer cell migration.

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DISCUSSION

To date, the class I_A isoform p110α is the only validated target of the PI3K family in the context of human cancer. Other class I_A isoforms have been associated with a role in various malignancies, including p110β in colon cancer, and p110δ in AML and breast cancer. Concerning the class II PI3Ks, PI3KC2α has been shown to play a role in survival of HeLa cells and increased expression of PI3KC2β was reported in a subset of tumours and cell lines from AML, GBM and SCLC (Arcaro et al., 2002; Knobbe & Reifenberger, 2003; Qian et al., 2002). Furthermore, PI3KC2β has been shown to play a role in the migration of A-431, HeLa and ovarian cancer cells, and to contribute to SCLC cell growth and Akt activation in response to growth factors such as SCF (Arcaro et al., 2002; Katso et al., 2006; Maffucci et al., 2005). In the present study, we have extended these studies on PI3KC2β in AML, brain tumours and neuroendocrine tumours.

IHere we report that PI3KC2β is overexpressed in a variety of human cancer cell lines and primary cultures compared to control tissue. The growing interest in small molecule inhibitors has led to the development of a plethora of pharmacological PI3K inhibitors whose activity in human cancer remains to be validated. In the present study, the effect of two specific inhibitors of PI3KC2β (PI701, PI702) on cancer cell responses was investigated. Upon treatment with either compound a dose-dependent inhibition of cell proliferation was observed in various human tumour cells while non-tumourigenic cells such as immortalised B cells or type II pneumocytes remained largely unaffected. Moreover, a correlation between protein expression levels and sensitivity to the inhibitors was observed in a number of cell lines. In AML, siRNA against PI3KC2β also led to decreased proliferation accompanied by an increase in apoptosis. The finding that the activation status of important signalling molecules including Akt, JNK and S6K was reduced upon treatment with PI701 is in line with the inhibitory effect of this compound on cell proliferation.

Interestingly, cotreatment of cells with PI701 led to an increased sensitivity to chemotherapeutic agents such etoposide and doxorubicin. This supports previous reports showing that the inhibition of important survival pathways, including PI3K/Akt, can enhance the response to cytotoxic reagents (Abdul-Ghani et al., 2006; Kumar et al., 2005). In the present study we have demonstrated for the first time that PI3KC2β inhibitors can sensitise human cancer cells to chemotherapeutic agents such as etoposide and doxorubicin.

Besides its role in regulating cell proliferation, in invasive murine breast cancer cell lines PI3KC2β was also found to be involved in cell migration. Enhanced expression of PI3KC2β was detected in the most motile cells (FibRas) as compared to non-invasive cells (EpH4). Treatment of these cells with increasing doses of PI701 strongly reduced the migratory capacity in wound healing assays. This is an interesting observation considering that malignant cells often acquire the ability to migrate leading to the propagation of metastases throughout the body.

In summary, the present study illustrates that PI3KC2β plays a crucial role in regulating various cellular responses in a broad spectrum of human cancer cells. Since the pharmacological inhibitor PI701 not only reduced basal cell proliferation but also had an effect on chemosensitivity and cellular migration, PI3KC2β could prove to be an attractive target for cancer treatment in the future.

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FIGURE LEGENDS

Figure 1 PI3KC2β expression in AML and SCLC cells. Western blot analysis of PI3KC2β expression in (**A**) normal bone marrow cells (N), primary blasts (1-7), AML cell lines and immortalized B cells (FIN COS, 41b MI) and (**B**) type II pneumocytes (PN) and SCLC cell lines (left panel). Relative mRNA expression levels of *PIK3C2B* in (**A**) human AML cell lines and (**B**) SCLC cell lines (right panel). Nontransformed type II human pneumocytes were used as a control.

Figure 2 PI3KC2β expression in tumours of the central nervous system. In (**A-B**) neuroblastoma cell lines and (**C-D**) primary tumour samples, PI3KC2β expression was analysed both by Western blot analysis (A, C) as well as quantitative RT-PCR (B, D). PI3KC2β protein expression in (**E**) glioblastoma cell lines and *ex vivo* cultures and (**F**) medulloblastoma cell lines by Western blot analysis. For neuroblastoma samples, human adrenal tissue (AG) was used as a control. For glioblastoma and medulloblastoma samples, normal human cerebellum served as a control.

Figure 3 Titration curve of PI701 in AML and SCLC. (**A**) AML cell lines (U937, triangles; HL-60, circles) and patient blasts (FAB M1, asterisks) and (**B**) SCLC cell lines (H209, triangles; H510, circles) were treated with increasing concentration of the inhibitor for 72 hours. For (**A**) AML cell lines and blast cells, immortalised B cells (diamonds and squares) served as a control. For (**B**) SCLC, nontransformed type II pneumocytes (squares) were used as a control.

Figure 4 Effect of PI701 on the phosphorylation of downstream signalling molecules. AML cell lines (U937 and THP1) were treated with increasing concentrations of PI701 overnight. Pathway activation was visualised by monitoring the phosphorylation status of Akt (P-AktSer^{473}), JNK (P-JNK$^{Thr183/Tyr185}$) and S6 protein (P-S6 protein$^{Ser235/236}$).

Figure 5 Inhibition of AML cell proliferation by small interfering RNA (siRNA) targeting PI3KC2β. (**A**) U937 cells transfected with control siRNA or siRNA targeting PI3KC2β were analyzed by western blotting for protein expression. (**B**) Cell proliferation of U937 cells transfected with siRNA targeting PI3KC2β was analysed by MTS assay (left panel) and caspase-3 activity was assessed in parallel. **P<0.01 by analysis of variance test.

Figure 6 PI701 sensitises AML and glioblastoma cells to chemotherapeutical agents. (**A**) Titration curve of U937 (left panel) or THP1 (right panel) cells incubated with increasing concentrations of etoposide in the absence (black circles) or presence (white circles) of PI701 (1μM). (**B**) Titration curve of T98G (left panel) or U251 (right panel) cells incubated with increasing concentrations of doxorubicin in the absence (black circles) or presence (white circles) of PI701 (1μM). (**C**) Titration curve of two glioblastoma *ex vivo* cultures incubated with increasing concentrations of doxorubicin in the absence (black circles) or presence (white

circles) of PI701 (1μM). A representative experiment (out of three) performed with eight repetitions is shown for A-C. *P<0.05 or **P<0.01 by analysis of variance test. (**D**) T98G cells were treated with PI701 or doxorubicin alone or in combination and caspase-3 cleavage was evaluated by means of a Western blot. (**E**) U937 cells were treated with increasing concentration of etoposide either alone or in combination with PI701 and PARP cleavage was visualised by means of a Western blot. The band corresponding to cleaved PARP (85 kDa) is shown.

Figure 7 Role of PI3KC2β expression in mammary epithelial cell lines. (**A**) Western blot analysis of PI3KC2β expression in EpH4, EpRas and FibRas cells. (**B**) Effect of PI701 on migration of mammary epithelial cell lines. Cells were treated with increasing concentrations of PI701 and the distance migrated was measured after 8 hours.

Supplemental Figure 1 Microarray analysis of PI3KC2β expression in a panel of primary medulloblastoma samples and cell lines. Gene expression profiles were obtained by using the Affymetrix HG-U133 Plus 2.0 array.

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Table I IC_{50} values (μM) against isolated enzymes.

Compound	PI3KC2β	p110α	p110β	p110γ	p110δ
PI701	**0.528**	**> 10**	**> 10**	**> 10**	**> 10**
PI702	**0.632**	**> 10**	**> 10**	**> 10**	**> 10**

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Table II IC_{50} values (μM) of PI701 and PI702 against a panel of cancer cell lines and primary cultures from NB, GBM, MB, AML, and SCLC.

IC50 Values		
	PI701	**PI702**
Neuroblastoma Cell Lines		
CHP134	7.4	8.0
LAN1	8.3	10.0
SHSY5Y	15.8	6.6
WAC2	5.2	6.0
Glioblastoma Cell Lines and *ex vivo* Cultures		
T98G	6.9	> 20
U251	8.0	> 20
LN319	19.6	14
LN229	6.2	14.1
U87	> 20	10.2
LO	11.6	ND
RE	13.2	ND
SB	18.2	ND
Medulloblastoma Cell Lines		
DAOY	10.1	> 20
D341	4.5	4.0
AML Cell Lines, Patient Blasts and Control Cells		
U937	3.2	5.5
HL-60	9.6	12.4
NB4	5.3	7.8
THP1	8.4	9.5
Kasumi	> 20	ND
KG-1	17.0	8.0
K562	10.4	> 20
FIN COS	> 20	> 20
41b MI	> 20	> 20
FAB M5	2.5	ND
FAB M1	3.6	ND
FAB M0	ND	6.2
N	ND	12.6
SCLC Cell Lines and Control Cells		
H69	4.4	ND
H209	4.3	ND
H510	5.0	ND
SW2	10.2	ND
PN	12.0	ND

LO, RE, SB = glioblastoma *ex vivo* cultures; FIN COS, 41b MI = immortalised B cells; FAB = French-American-British classification; N = non-leukemic bone marrow cells; PN = immortalised Type II pneumocytes; ND = not determined

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Figure 1

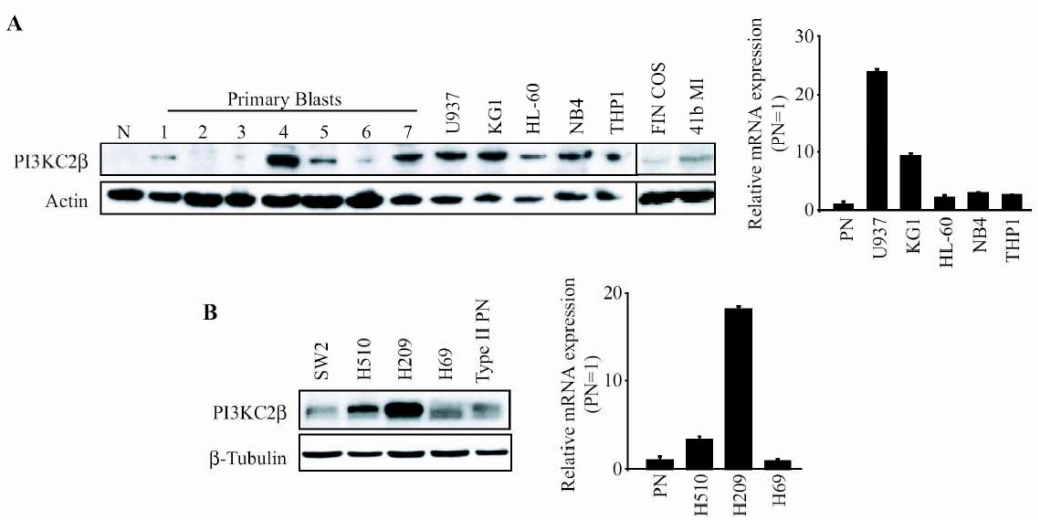
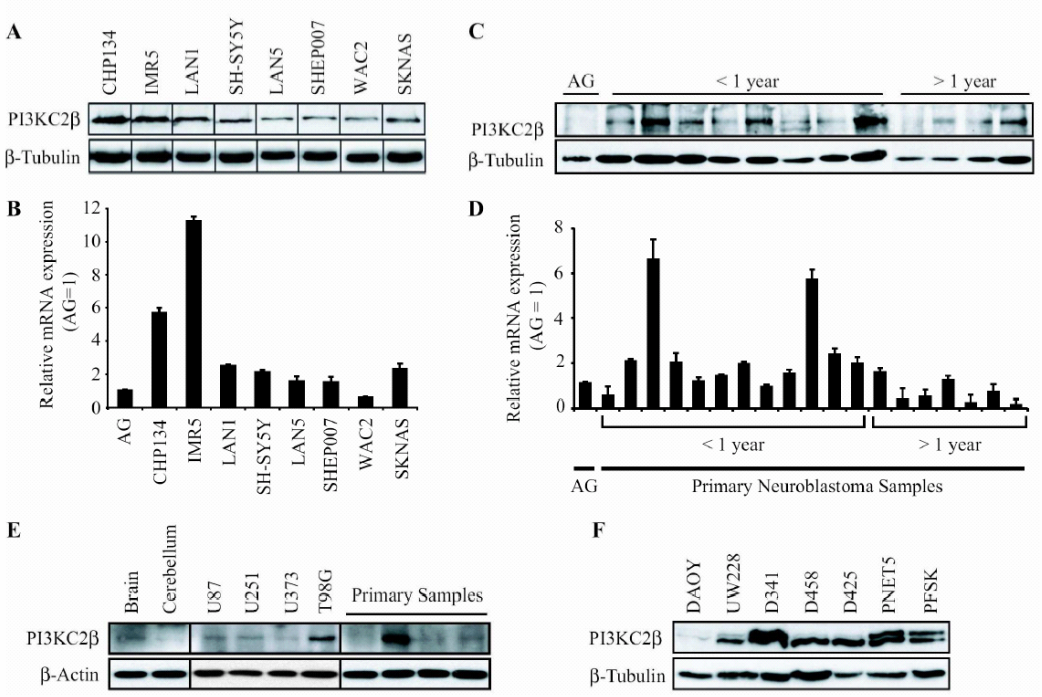
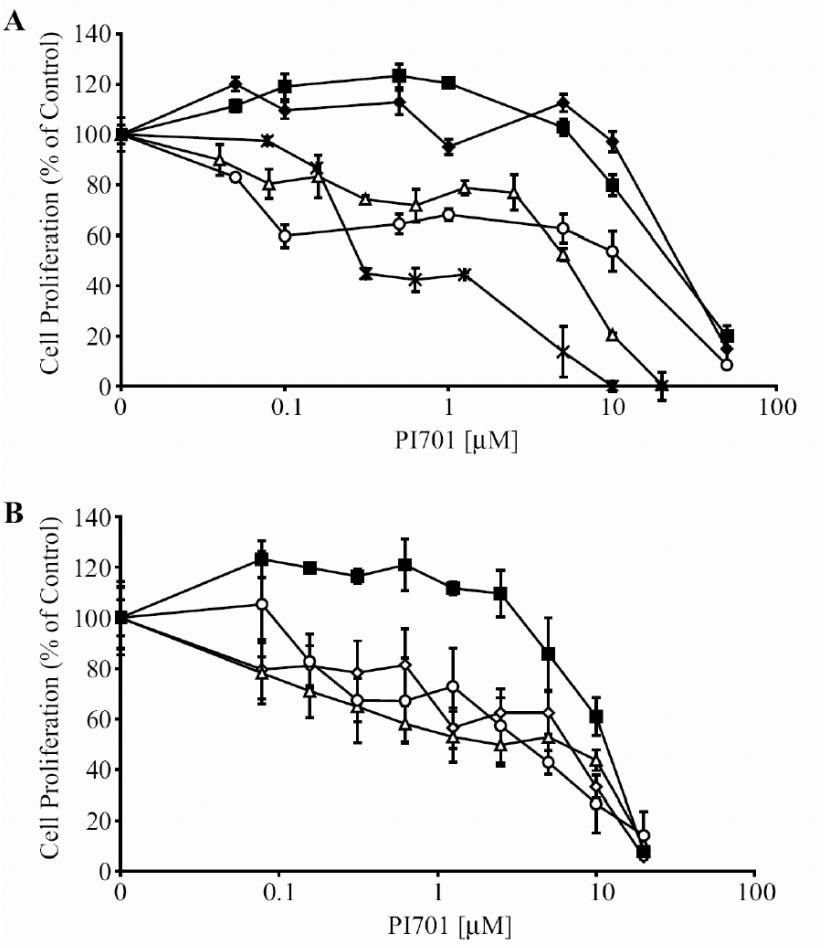

A
Primary Blasts
N 1 2 3 4 5 6 7
U937 KG1 HL-60 NB4 THP1 FIN COS 41b MI
PI3KC2β
Actin
Relative mRNA expression (PN=1)
30
20
10
0
PN U937 KG1 HL-60 NB4 THP1
B
SW2 H510 H209 H69 Type II PN
PI3KC2β
β-Tubulin
Relative mRNA expression (PN=1)
20
10
0
PN H510 H209 H69

Figure 2


A
CHP134
IMR5
LAN1
SH-SY5Y
LAN5
SHEP007
WAC2
SKNAS
PI3KC2β
β-Tubulin
C
AG
< 1 year
> 1 year
PI3KC2β
β-Tubulin
B
Relative mRNA expression (AG=1)
12
10
8
6
4
2
0
AG
CHP134
IMR5
LAN1
SH-SY5Y
LAN5
SHEP007
WAC2
SKNAS
D
Relative mRNA expression (AG = 1)
8
6
4
2
0
< 1 year
> 1 year
AG
Primary Neuroblastoma Samples
E
Brain
Cerebellum
U87
U251
U373
T98G
Primary Samples
PI3KC2β
β-Actin
F
DAOY
UW228
D341
D458
D425
PNET5
PFSK
PI3KC2β
β-Tubulin

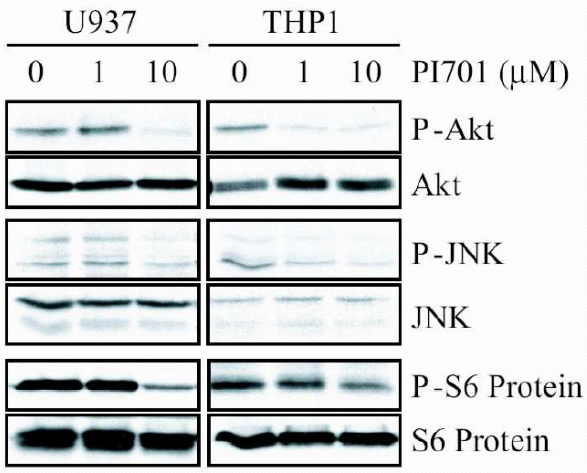
21

Figure 3


A
Cell Proliferation (% of Control)
140
120
100
80
60
40
20
0
0
0.1
1
10
100
PI701 [µM]
B
Cell Proliferation (% of Control)
140
120
100
80
60
40
20
0
0
0.1
1
10
100
PI701 [µM]

22

Figure 4


U937
THP1
0 1 10 0 1 10 PI701 (μM)
P-Akt
Akt
P-JNK
JNK
P-S6 Protein
S6 Protein

23

Figure 5

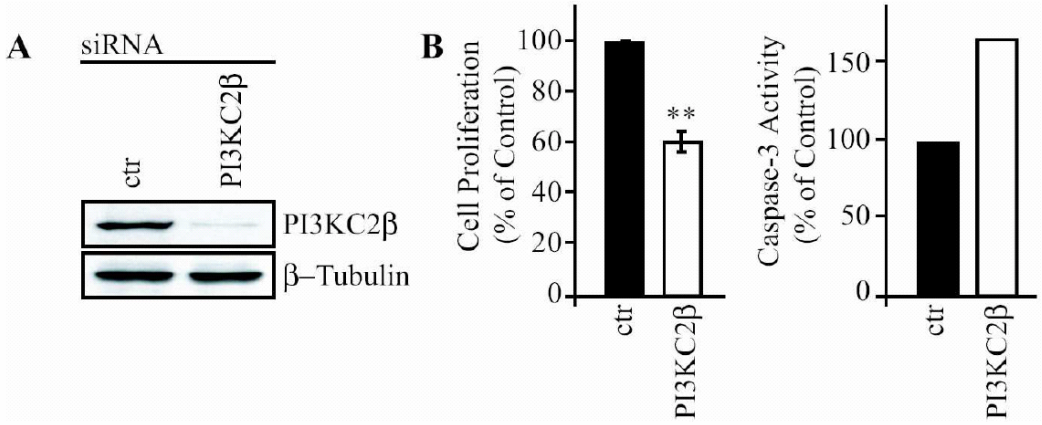
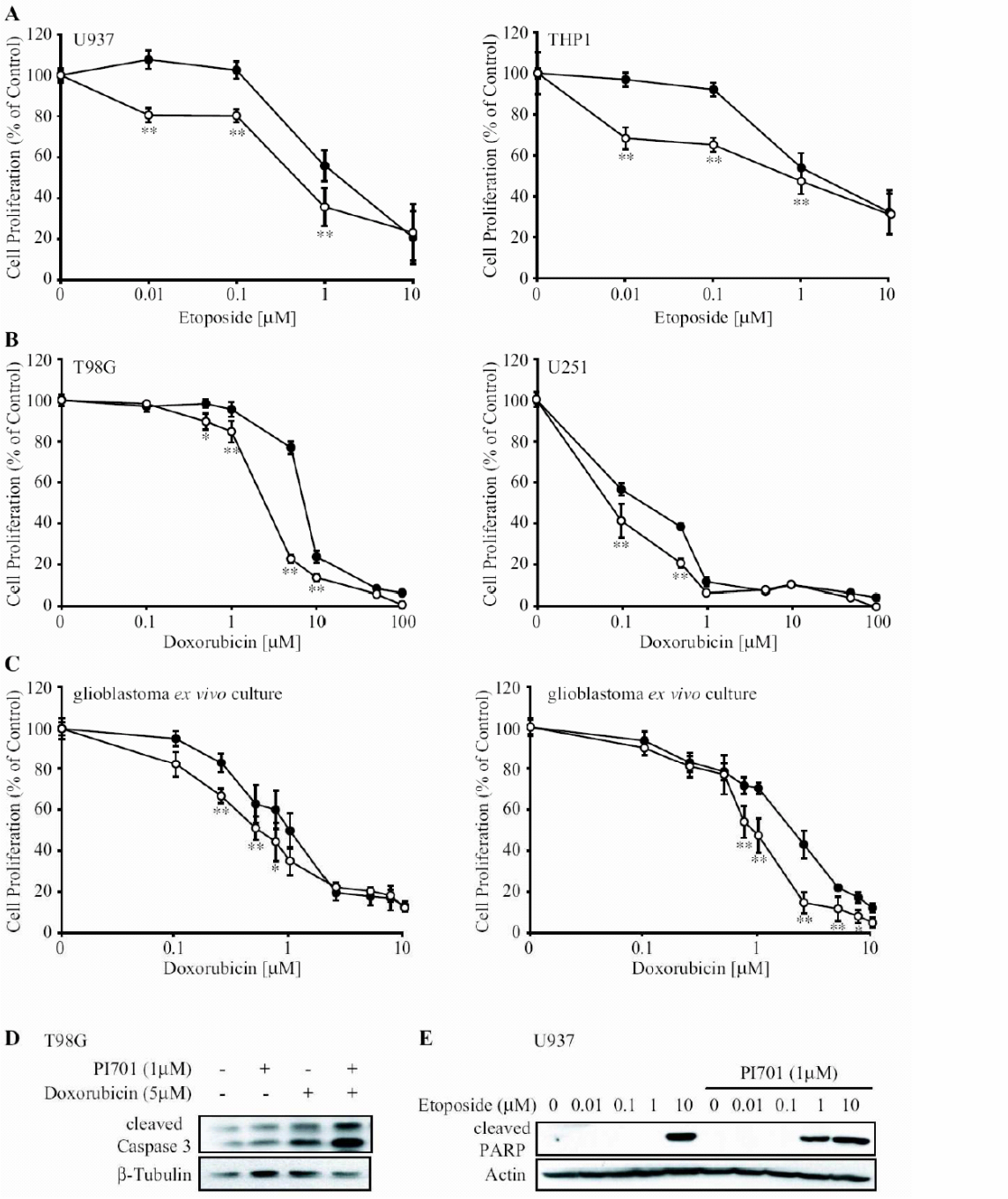
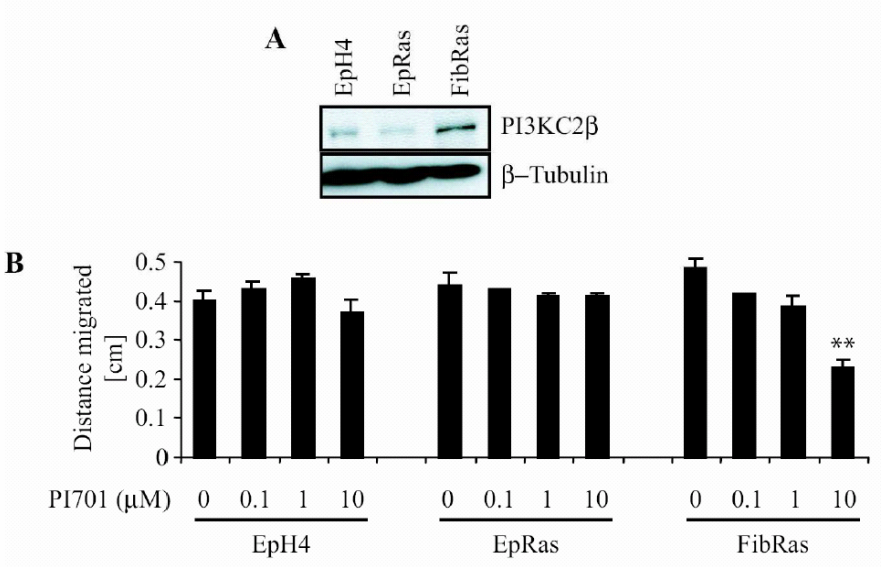

A
siRNA
ctr
PI3KC2β
PI3KC2β
β–Tubulin
B
Cell Proliferation (% of Control)
100
80
60
40
20
0
**
ctr
PI3KC2β
Caspase-3 Activity (% of Control)
150
100
50
0
ctr
PI3KC2β

Figure 6

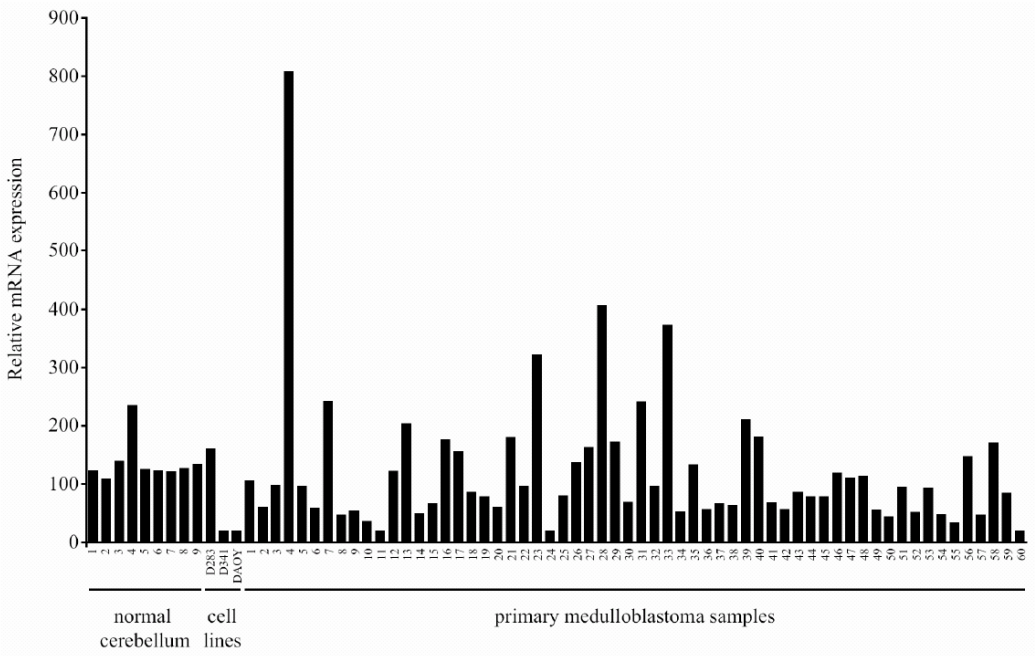

A
U937
THP1
Cell Proliferation (% of Control)
Etoposide [µM]
B
T98G
U251
Doxorubicin [µM]
C
glioblastoma ex vivo culture
D
T98G
PI701 (1µM)
Doxorubicin (5µM)
cleaved Caspase 3
β-Tubulin
E
U937
PI701 (1µM)
Etoposide (µM) 0 0.01 0.1 1 10 0 0.01 0.1 1 10
cleaved PARP
Actin

25

Figure 7


A
EpH4
EpRas
FibRas
PI3KC2β
β–Tubulin
B
Distance migrated [cm]
0.5
0.4
0.3
0.2
0.1
0
**
PI701 (μM) 0 0.1 1 10 0 0.1 1 10 0 0.1 1 10
EpH4
EpRas
FibRas

Supplemental Figure 1



3.2.5 Distinct class I_A PI3K isoforms regulate glioblastoma cell growth, survival and migration *(Manuscript in preparation)*

Distinct class I_A PI3K isoforms regulate glioblastoma cell growth, survival and migration

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ABSTRACT

Glioblastoma (GBM) is a common malignant form of brain tumor associated with poor prognosis. The phosphoinositide 3-kinase (PI3K)/Akt pathway is frequently activated in GBM due to mutations in the tumor suppressor gene phosphatase and tensin homologue (*PTEN*), the *PIK3CA* gene encoding the PI3K isoform p110α or due to mutation or over-expression of receptor tyrosine kinases. In the present study, the expression and functions of the catalytic class I_A PI3K isoforms were investigated in GBM cell lines and *ex vivo* cultures. Targeting the isoforms p110β or p110δ using RNA interference (RNAi) or isoform-specific inhibitors did not significantly impair the growth of GBM cells under normal culture conditions, while inhibition of p110α had a partial effect. However, the isoforms p110α and p110β contributed to GBM growth under anchorage-independent conditions. We further observed a selective role for p110β in protecting GBM cells from anoikis (detachment-induced apoptosis). In addition, p110β was essential for the ability of GBM cells to migrate, while p110α and p110δ were dispensable for the response. The p110α isoform also appeared to play a role in the chemosensitivity of GBM cells towards doxorubicin and cisplatin. Moreover, p110α was essential for RTK coupling to the downstream effectors Akt and ribosomal protein S6 kinase (S6K). Together, these results uncover distinct functions for the catalytic class I_A PI3K isoforms p110α and p110β in GBM cell responses.

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INTRODUCTION

Gliomas are the most common primary central nervous system (CNS) tumors, and glioblastoma (GBM) represent the largest subgroup (1). Amongst the brain tumors, glioblastoma are associated with poor survival, due to tumor cell resistance to radiotherapy and chemotherapy, and infiltration of normal brain tissue (2). Polypeptide growth factors have been shown to play a key role in GBM proliferation, chemoresistance/radioresistance and metastasis. Genetic alterations involving the epidermal growth factor receptor (EGFR) are commonly found in GBM. A characteristic mutation leads to the generation of the mutant receptor subtype EGFRvIII (3), whose expression has been shown to correlate with elevated Akt activation (4). In GBM, signaling via the EGFR has also been demonstrated to contribute to resistance to radiation and chemotherapy (5). Other studies have also described a role for hepatocyte growth factor (HGF) and insulin-like growth factor-I (IGF-I) in human GBM chemoresistance (6, 7). Moreover, platelet-derived growth factor (PDGF) has been shown to signal in an autocrine fashion in GBM cells, thus contributing to proliferation and survival of tumor cells (8). Fibroblast growth factor-2 (FGF-2) is also expressed in human GBM (9, 10) and may have chemoprotective and radioprotective effects, considering that anti-apoptotic functions have been described in other human cancers (11). Therefore, given that GBM cells express a variety of different growth factor receptors, targeting individual receptors may not necessarily provide a successful therapeutic strategy. An alternative approach would be to identify a signaling molecule that lies downstream of several different growth factor receptors and plays an essential role in transmitting their proliferative and/or survival signals.

Phosphoinositide 3-kinase (PI3K) is a good example of such a molecule, in view of the fact that it plays a crucial role in controlling cell proliferation, survival and motility/metastasis downstream of many different growth factor receptors. PI3Ks phosphorylate phosphatidylinositol (PI) on the D-3 position of the inositol ring, producing three distinct second messengers: PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 (PIP_3). The importance of PI3K signaling in cancer is highlighted by the fact that mutations in the tumor suppressor gene phosphatase and tensin homologue (*PTEN*) occur frequently in human tumors. PTEN is a phosphatase that antagonizes the action of PI3K by de-phosphorylating the D-3 position of polyphosphoinositides (12, 13). Moreover, recent reports have described activating mutations in the *PIK3CA* gene, which encodes the catalytic p110α isoform of PI3K, in a variety of human malignancies including, breast, colon, ovarian and brain cancers (14-16).

The importance of PI3K signaling in GBM is highlighted by the fact that mutations in *PTEN* occur with high frequency and have prognostic value (17). PI3K signaling has also been demonstrated to mediate chemoresistance of GBM induced by polypeptide growth factors such as epidermal growth factor (EGF), HGF and IGF-I (6, 7, 18). Moreover, recent reports have described activating mutations in the *PIK3CA* gene (16). Amplification of other genes encoding PI3K isoforms has also been reported in human GBM (19, 20). In addition, over-expression of *PIK3CD* mRNA (encoding p110δ) was

reported in some cases of GBM (20). Thus, targeting the PI3K pathway may represent an attractive novel approach to develop therapies for GBM (5, 21). Indeed, several recent reports have described that experimental approaches targeting components of the PI3K/Akt pathway induce apoptosis, inhibit GBM growth *in vitro* and *in vivo,* and sensitize the tumor cells to chemotherapy, radiotherapy and RTK inhibitors (22-28). A recent study could also show that a dual inhibitor of p110α and mTOR displayed remarkable anti-tumor activity against glioma cells *in vivo* (29). Taken together, deregulation of the PI3K/Akt pathway is frequently detected in GBM and has been found to contribute to a variety of cellular responses, thus making this pathway an interesting candidate for targeted therapies.

RESULTS

Over-expression of PI3K isoforms in human GBM cell lines and* ex vivo *cultures. Initial experiments were aimed at gaining an overview of the expression pattern of class I_A PI3K isoforms in human GBM cell lines and *ex vivo* (EV) cultures. We further analyzed the basal activation of PI3K/Akt signaling components by assessing their phosphorylation status. Protein expression levels in GBM samples were compared to normal human brain or cerebellum tissue as well as to immortalized type II pneumocytes. While expression of the regulatory subunit p85α was comparable to levels observed in control lysates, differences in expression of the catalytic subunits p110α, p110β and p110δ were found (Fig. 1*A*). p110α showed the most striking variation with some samples showing over-expression (U251, T98G, EV5), while expression of this isoform was nearly absent in certain cell lines (LN215, LN229, LN319) and *ex vivo* cultures (EV1, EV2, EV3, EV4, EV6, EV7) (Fig. 1*A*). Expression of p110β was largely comparable to levels detected in control samples (Fig. 1*A*). Interestingly, p110δ was over-expressed in more than half of all glioblastoma cell lines analyzed (LN319, U87, U251, U373), while the expression levels observed in *ex vivo* cultures were comparable to the controls (Fig. 1*A*). Basal activation of Akt was detected more frequently in cell lines (LN319, U87, U251, U373, T98G) than in *ex vivo* cultures (EV5, EV7) when compared to control samples (Fig. 1*A*). The most striking difference was found upon analysis of S6 protein activation. Strong basal phosphorylation of this protein was observed in all GBM cell lines and *ex vivo* cultures analyzed when compared to control lysates (Fig. 1*A*). Taken together, this expression analysis revealed that individual catalytic class I_A PI3K isoforms are over-expressed in GBM cell lines and *ex vivo* cultures. Moreover, basal activation of the PI3K/Akt/S6 pathway was detected in the samples analyzed. While phosphorylation of Akt was only observed in a subset of GBM cell lines and *ex vivo* cultures, activation of S6 protein was observed in all samples included in this study. These results indicate that the PI3K/Akt/S6 pathway is aberrantly activated in GBM.

Pharmacological inhibition of p110α impairs cell proliferation and leads to decreased phosphorylation of Akt and S6. In recent years, numerous attempts have been made to develop isoform-specific PI3K inhibitors (30-34). These efforts have indeed led to the generation of compounds that exhibit increased specificity towards individual isoforms. In the present study, we used specific inhibitors of p110α (YM024 (35)), p110β (TGX-221 (36)) and p110δ (IC87114 (37)) to evaluate the contribution of these isoforms to cellular responses. Two human GBM cell lines (T98G and U251) and two human *ex vivo* cultures (EV6 and EV7) were treated with increasing concentrations of each inhibitor. While inhibition of p110β or p110δ did not have a significant impact on cell proliferation in any of the cells investigated (Fig. 2*C* and 2*D*), inhibition of p110α led to decreased proliferation in both cell lines and *ex vivo* cultures (Fig. 2*A*). Western blot analysis of the activation status of downstream signaling molecules of the PI3K/Akt/S6 pathway revealed a dose-

dependent inhibition of Akt and S6 phosphorylation upon treatment with YM024 in T98G and U251 cells (Fig. 2*B*). This is in line with the observed effects on proliferation. These data suggest that the p110α isoform plays a crucial role in regulating basal proliferation of GBM cells, while inhibition of p110β or p110δ did not have a comparable effect.

p110β is involved in regulating anchorage-independent growth and cell migration. Various studies have reported a contribution of p110β to anchorage-independent growth of different cell systems (38, 39). We therefore sought to analyze the effects of specifically inhibiting the catalytic class I_A PI3K isoforms in regard to colony formation. T98G and U251 cells were treated with increasing concentrations of YM024, TGX-221 or IC87114 and the number of colonies per well was then evaluated. Inhibition of p110α negatively influenced colony formation in both cells lines at 1μM (Fig. 3*A*; T98G: 70% inhibtion, U251: 39% inhibition). The effect of p110β inhibition was more striking, since 0.1μM TGX-221 was sufficient to significantly reduce the number of visible colonies (Fig. 3*A*; T98G: 36% inhibition, U251: 54% inhibition). Interestingly, the number of colonies increased slightly at higher concentrations of the p110β inhibitor (Fig. 3*A*, TGX-221 1μM). We further analyzed the effect of siRNA-mediated PI3K isoform downregulation on anoikis in T98G cells. While downregulation of p110α or p110δ did not greatly affect anoikis (Fig. 3*B*, PIK3CA and PIK3CD), siRNA-targeting of p110β led to a 15% increase in detachment-induced cell death (Fig. 3*B*, PIK3CB). To complement these studies, we also analyzed the contribution of individual isoforms to the motility of T98G cells. Our results show that downregulation of p110β negatively affects cell speed (Fig. 3*C*, PIK3CB) when compared to control cells (Fig. 3*C*, scrambled), while siRNA-targeting of p110α or p110δ had no significant effect (Fig. 3*C*, PIK3CA and PIK3CD). Analysis of the distance covered by T98G cells transfected with siRNA against p110β (Fig. 3*D*, PIK3CB) revealed a highly restricted area of movement when compared to mock-transfected cells (Fig. 3*D*, scrambled). Thus, p110β seems to contribute significantly to the motility of GBM cells.

Pharmacological inhibition of p110α sensitizes GBM cell lines and* ex vivo *cultures to chemotherapeutic agents. We next investigated whether pharmacological inhibition of p110α can modulate the sensitivity of GBM cell lines and *ex vivo* cultures to chemotherapeutic agents. Two GBM cell lines (T98G and U251) as well as one *ex vivo* culture were treated with doxorubicin or cisplatin alone or in combination with 1μM YM024. We observed increased sensitivity to the DNA-damaging agents when used in combination with the p110α inhibitor, compared to single-agent treatment (Fig. 4*A-C*). These observations are in line with previous reports demonstrating that co-treatment of cells with the broad-spectrum PI3K inhibitors LY294002 and wortmannin increases their sensitivity to chemotherapeutic agents (40-42). A growing body of evidence suggests that that activation of the PI3K/Akt signaling pathway plays an important role in conferring broad-spectrum chemoresistance to

cancer cells (28, 40, 43). Considering that genetic alterations involving *PTEN* are frequent events in GBM and are associated with elevated Akt activity (44), it is conceivable that the abrogation of basal Akt and S6 protein phosphorylation induced by treatment with YM024 contributes to the observed chemosensitization in GBM cells.

Growth factor mediated activation of the PI3K/Akt pathway is attenuated upon pharmacological inhibition of p110α. The list of growth factors implicated in the biology of GBM is long. Numerous receptor tyrosine kinases (RTKs) are thought to contribute to the malignant properties of gliomas through autocrine signaling loops. Well documented growth factor systems include the EGF system (45), the PDGF system (46, 47) and the IGF system (48-50). Based on the observation that pharmacological inhibition of p110α efficiently attenuated basal PI3K/Akt activation, we were interested in evaluating the potential of YM024 to inhibit growth factor-induced pathway activation. Indeed, pre-treatment of T98G cells and an *ex vivo* GBM culture impaired phosphorylation of Akt and S6 protein in response to EGF, PDGF or IGF-I stimulation (Fig. 5*A*). These results implicate that p110α plays a crucial role in transmitting signals from activated RTKs and thus could be involved in autocrine signaling events in GBM.

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DISCUSSION

Studies performed *in vitro* and *in vivo* have provided compelling evidence that targeting the PI3K/Akt pathway can lead to suppression of proliferation as well as increased sensitivity to chemotherapeutic agents in GBM (27, 29, 42, 51). Moreover, this pathway also contributes significantly to the migratory and invasive properties of GBM cells (21, 52). Previous studies targeting the PI3K/Akt pathway have used ectopic expression of wild type PTEN in *PTEN*-deficient GBM cell lines (44), generic PI3K inhibitors such as wortmannin or LY294002 (27, 53), as well as the inhibitor PI-103 (29, 54). Although initially claimed to be selective for p110α and mTOR, subsequent work found that the latter compound also inhibits the other catalytic class I PI3K isoforms, as well as other enzymes related to PI3K (54). Therefore, the exact contribution of class I PI3K isoforms to GBM cell responses remains to be established. In the present study, we have used isoform-specific PI3K inhibitors and RNAi to further elucidate the contribution of the three catalytic class I_A PI3K isoforms p110α, p110β and p110δ to GBM cell proliferation, survival and migration. We initially assessed the relative expression of these isoforms in cell lines and *ex vivo* cultures and found that the p110β and p110δ were consistently over-expressed in GBM cultures, as compared to normal brain, while the expression levels of p110α were more variable. Upon individual inhibition of the three catalytic class I_A PI3K isoforms, limited effects on cell proliferation under normal culture conditions were observed with the p110α inhibitor. However, when anchorage-independent growth of GBM cells was investigated, the contribution of p110α and p110β became more apparent, indicating that PI3K/Akt signaling plays a more important role under limiting culture conditions. In support of this notion, detachment-induced apoptosis of GBM cells was significantly enhanced upon siRNA-mediated targeting of p110β. Intriguingly, chemotherapy-induced apoptosis was selectively enhanced by targeting p110α, while inhibition of p110β did not affect the response. Together, these results indicate that p110α and p110β differentially regulate anti-apoptotic signaling in GBM cells depending on the stress involved. In view of the major role p110β plays in regulating the migratory response of the these cells, it can be speculated that this isoform plays a crucial role in cytoskeletal rearrangements in GBM cells.

Together, the data presented in the present report highlight the different roles of the catalytic class I_A PI3K isoforms p110α and p110β in GBM cells responses, and provides a rationale for the future design of combinatorial drug therapies for this aggressive type of cancer.

MATERIALS AND METHODS

Reagents and Antibodies

Antibodies were purchased from the following companies: p85α, p110β, p110δ, Akt/PKB - Santa Cruz Biotechnology; p110α, S6 protein and phosphospecific antibodies for Akt/PKB (Ser473; Thr308) and S6 protein (Ser235/236; Ser240/244) - Cell Signaling Technology; β-Actin and Cisplatin - Sigma-Aldrich. Doxorubicin was purchased from Pfizer AG; EGF, PDGF and IGF-I were obtained from Calbiochem. YM024 and TGX-221 were generously provided by Prof. SP Jackson and IC87114 was received from ICOS Corporation.

Anoikis Assay

These assays were performed as described (55).

Cell Culture

Cell lines were grown in DMEM (Life Technologies/Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin/streptomycin/L-glutamine and passaged every 3-5 days by trypsinization. *Ex vivo* glioblastoma cultures were kept in DMEM (Life Technologies/Invitrogen) supplemented with 10% (v/v) heat inactivated FCS and gentamycin/L-glutamine.

Cell Proliferation

Cells were seeded in 96-well plates at a density of 5000 cells per well and grown for 72h in serum (10%)-containing medium in the presence or absence of inhibitors or chemotherapeutic agents as indicated. The number of viable cells was analyzed by means of an MTS assay using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Data are mean with SD from 8 repetitions.

Dissociation of Brain Tumors

Human brain tumors were removed from 4 patients who underwent surgery for tumor resection at the University Hospital of Zurich. All procedures were conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Canton Zurich. Following removal, tumor tissue was immediately placed in a petri dish, minced mechanically and digested enzymatically with collagenase D and DNase I (Roche Applied Science) for 1 hour at 37°C while being stirred with a magnetic bar. The dissociated cells were then sequentially filtered through 100µm and 70µm cell strainers (BD Falcon, BD Biosciences) to remove any tissue debris. Erythrocytes were removed by resuspending and incubating the cells in ice-cold ACK buffer (17 mM Tris–HCl [pH 7.2] containing 144 mM NH_4Cl) for 10 minutes on ice. The cells were washed in PBS and plated in DMEM (Life

Technologies/Invitrogen) supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS) and gentamycin (20mg/ml) and passaged every 3-5 days by trypsinization.

Growth Factor Stimulations

Cells were grown to confluency in a 6-well plate and starved overnight in DMEM containing 0.5% FCS. Cells were maintained in serum-free DMEM for 1 hour in the presence or absence of inhibitors as specified and were then stimulated with the indicated growth factors for 10 minutes. Cellular lysates were prepared as previously described (56).

Migration Assay

These assays were performed as described (55).

SDS-PAGE and Western blot analysis

Cellular lysates were prepared as described (56) and normalized using a bicinchoninic acid (BCA) protein assay (Pierce). Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Biosciences) and immunoblotted with the indicated antibodies prior to chemiluminescent detection (ECL Western blotting detection reagents; Amersham Biosciences).

siRNA-mediated Downregulation of PI3K Isoform Expression

These assays were performed as described (55).

Soft Agar Assay

Soft agar colony formation assays were performed in a 6-well format with T98G and U251 glioblastoma cell lines. 500 cells per well were cultured in 0.35% top agar supported by a 0.5% layer of base agar in the medium described above. Plates were incubated at 37°C for 21-28 days. The number of visible colonies (>1mm) was assessed by staining plates with 0.05% crystal violet for 1h.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1

Expression pattern of molecules involved in PI3K signaling. (A) Protein levels of class IA PI3K isoforms and activation of the downstream signaling molecules Akt and S6 protein were assessed in human GBM cell lines (*left panel*) and *ex vivo* cultures (*right panel*) by means of Western blot analysis. Normal human brain and cerebellum tissue, as well as non-transformed type II human pneumocytes were used as controls. β-Actin served as a loading control.

Figure 2

Pharmacological inhibition of p110α decreases proliferation of GBM cell lines and *ex vivo* cultures and attenuates basal activation of Akt and S6 protein. Two GBM cell lines (T98G and U251) and two *ex vivo* cultures (EV6 and EV7) were treated with increasing concentrations of YM024, an isoform-specific inhibitor of p110α. (A) Basal proliferation in complete medium was assessed by means of an MTS assay after 72h. (B) Pathway activation was monitored by visualizing the phosphorylation status of Akt and S6 protein. (C-D) The effect of TGX221 (a p110β inhibitor) as well as IC87114 (a p110δ inhibitor) on basal proliferation was assessed after 72h by means of an MTS assay. These inhibitors had a less marked impact on basal proliferation than the p110α inhibitor YM024.

Figure 3

A role for the class IA PI3K isoform p110β in colony formation and induction of anoikis. (A) T98G (*left panel*) and U251 (*right panel*) cells were grown in soft agar in the presence of increasing concentrations of isoform-specific PI3K inhibitors as indicated. Pharmacological inhibition of p110β was found to inhibit colony formation at low concentrations (0.1μM). Inhibition of p110α also led to a decrease in colony numbers at concentrations compatible with specific inhibition of this protein (1μM). (B) Analysis of anoikis following siRNA-mediated downregulation of PI3K expression, as assessed by changes in SubG1 cell populations. Decreased expression of p110β (PIK3CB) was found to induce anoikis in T98G cells, while downregulation of p110α (PIK3CA) or p110δ (PIK3CD) had little or no effect compared to control cells (scrambled). (C) Migration speed in T98G cells following downregulation of class IA PI3K isoforms. Decreasing the levels of p110β (PIK3CB) was found to significantly reduce the migratory speed of T98G cells, while decreased levels of p110α (PIK3CA) or p110δ (PIK3CD) had no significant impact. (D) Distance of migration in T98G cells following siRNA-mediated downregulation of p110β. The area covered by cells expressing decreased levels of p110β (PIK3CB) was restricted compared to control cells (scrambled).

Figure 4

The class IA PI3K isoform p110α is involved in regulating chemosensitivity in GBM cell lines and *ex vivo* cultures. Two human GBM cell lines (T98G (A) and U251 (B)) and an *ex vivo* culture (EV7 (C)) were treated with increasing concentrations of doxorubicin or cisplatin either in the absence (black squares) or presence (open circles) of 1μM YM024. Pharmacological inhibition of p110α sensitized T98G and EV7 cells to doxorubicin, while U251 cells displayed increased sensitivity to cisplatin upon concomitant treatment with YM024.

Figure 5

Pharmacological inhibition of p110α impairs PI3K/Akt pathway activation upon growth factor stimulation. (A) A human GBM cell line (T98G) and an *ex vivo* culture (EV7) were pre-treated with 1μM YM024 for 1h. Pathway activation was monitored by assessing the phosphorylation status of Akt and S6 protein. Pre-treatment with YM024 reduced basal levels of phosphorylated Akt and S6 protein and attenuated pathway activation upon stimulation with growth factors. YM024 had the strongest effect on pathway activation induced by PDGF or IGF-I.

Supplemental Figure 1

Age and sex of patients as well as WHO grade of gliomas, from which *ex vivo* cultures were established.

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FIGURES

Figure 1

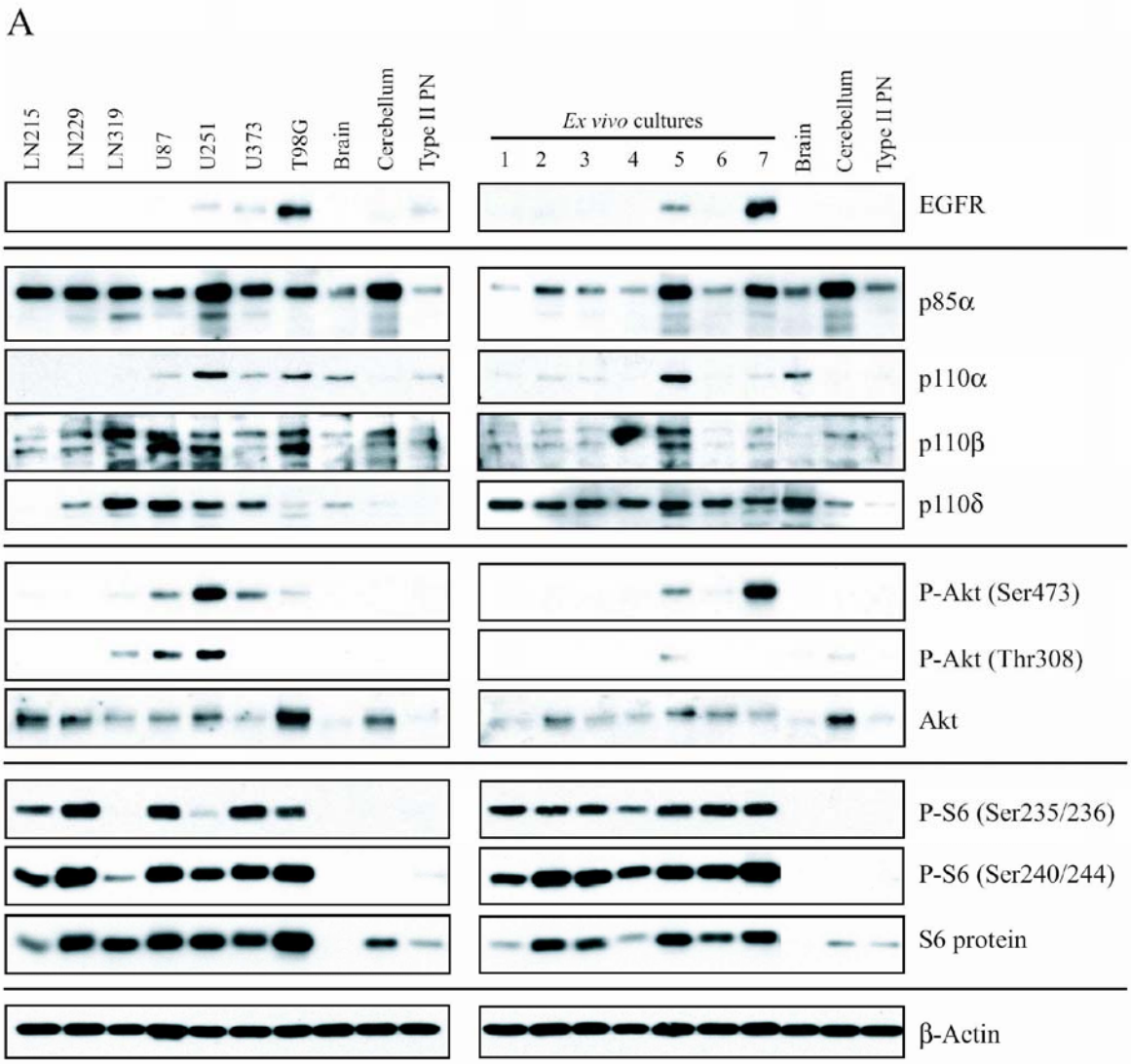
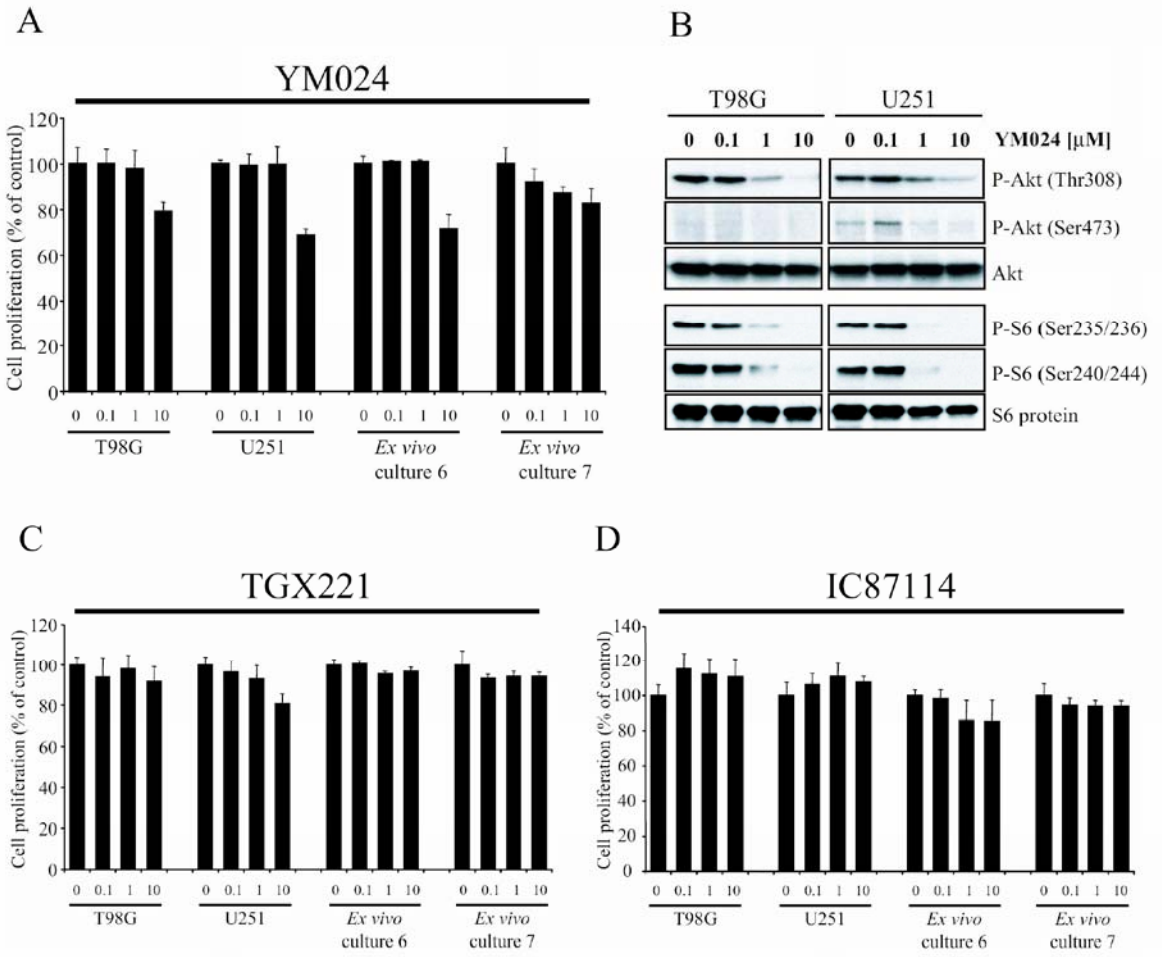
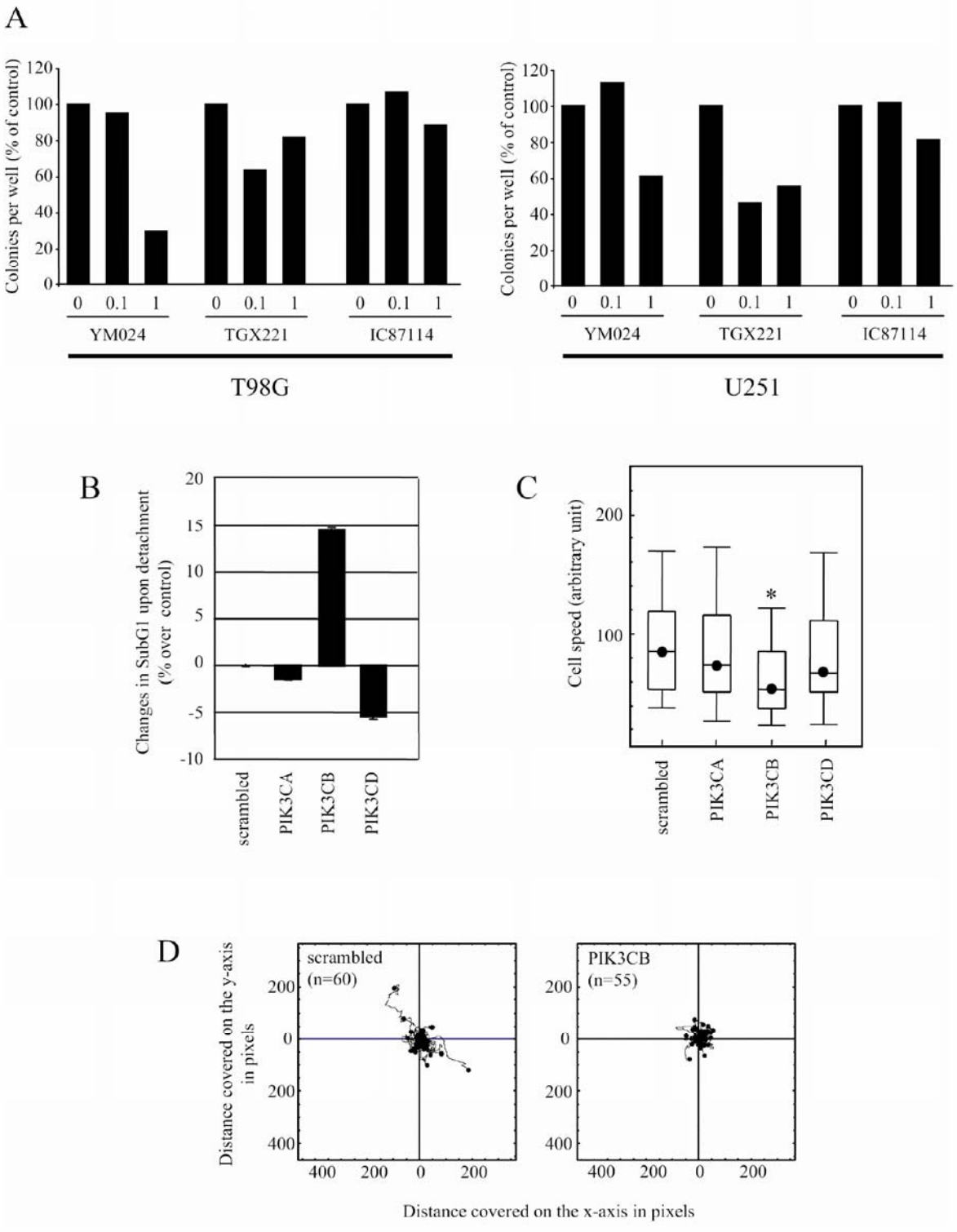


Figure 2


A
YM024
Cell proliferation (% of control)
120
100
80
60
40
20
0
0 0.1 1 10
T98G
U251
Ex vivo culture 6
Ex vivo culture 7
B
T98G
U251
0 0.1 1 10
YM024 [μM]
P-Akt (Thr308)
P-Akt (Ser473)
Akt
P-S6 (Ser235/236)
P-S6 (Ser240/244)
S6 protein
C
TGX221
D
IC87114
140

18

Figure 3


A
Colonies per well (% of control)
120
100
80
60
40
20
0
0 0.1 1 0 0.1 1 0 0.1 1
YM024
TGX221
IC87114
T98G
U251
B
Changes in SubG1 upon detachment (% over control)
20
15
10
5
0
-5
-10
scrambled
PIK3CA
PIK3CB
PIK3CD
C
Cell speed (arbitrary unit)
200
100
*
D
Distance covered on the y-axis in pixels
scrambled (n=60)
PIK3CB (n=55)
200
0
200
400
400 200 0 200
Distance covered on the x-axis in pixels

19

Figure 4

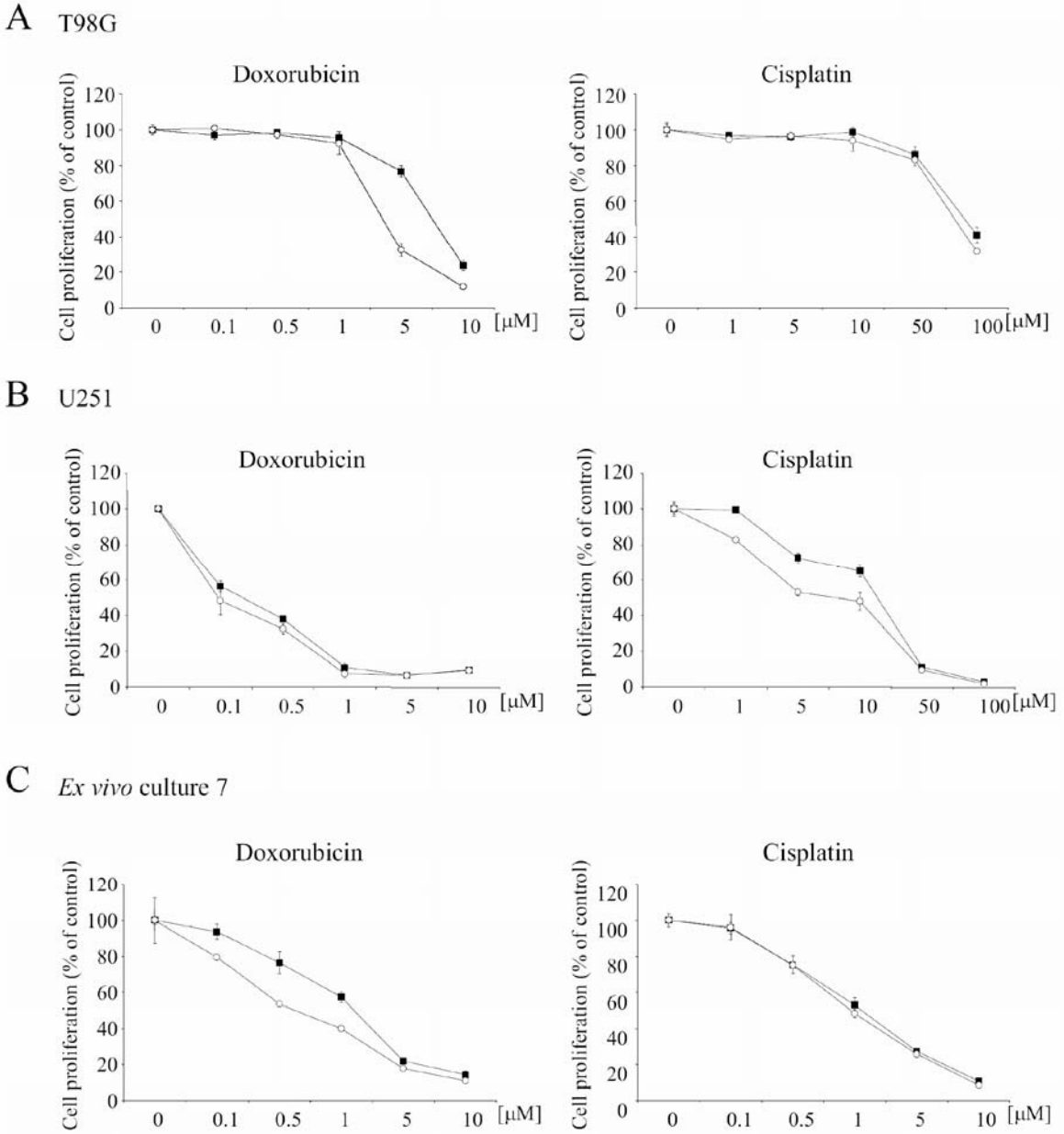
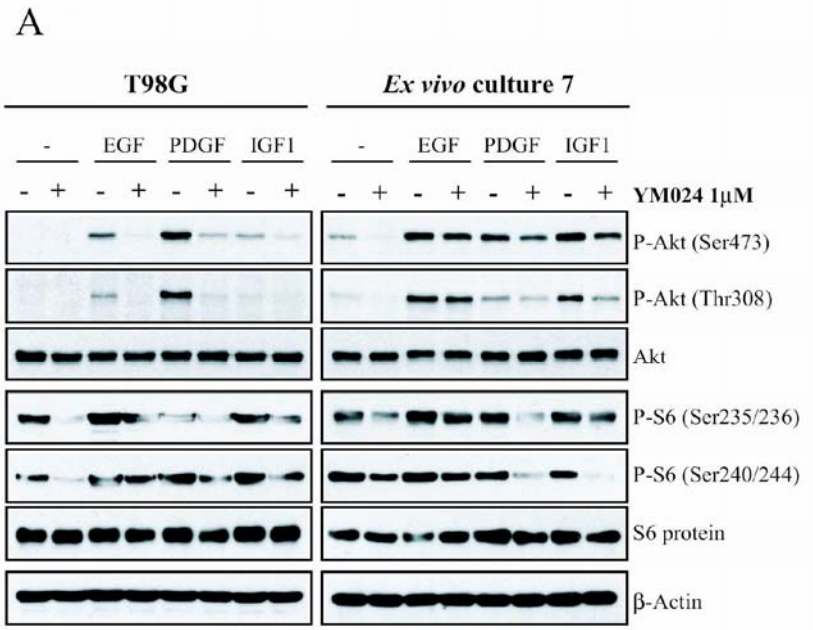

A T98G
Doxorubicin
Cisplatin
Cell proliferation (% of control)
120
100
80
60
40
20
0
0 0.1 0.5 1 5 10 [μM]
0 1 5 10 50 100 [μM]
B U251
Doxorubicin
Cisplatin
Cell proliferation (% of control)
0 0.1 0.5 1 5 10 [μM]
0 1 5 10 50 100 [μM]
C Ex vivo culture 7
Doxorubicin
Cisplatin
Cell proliferation (% of control)
0 0.1 0.5 1 5 10 [μM]
0 0.1 0.5 1 5 10 [μM]

Figure 5



Supplemental Figure 1

	WHO Grade	Age [years]	Sex
EV1	III	37	F
EV2	IV	77	M
EV3	II	42	F
EV4	IV	59	M
EV5	III	61	M
EV6	IV	65	M
EV7	IV	54	M

4 Conclusions and Future Perspectives

4.1 Conclusions

The aim of this dissertation was to investigate how the family of PI3Ks contributes to the biology of NB and GB. Analysis of primary tumor samples was complemented by experiments performed in cell culture, allowing a more detailed investigation of molecular pathways. Two important tools used in the described studies included a novel generation of pharmacological isoform-specific PI3K inhibitors as well as siRNA- or short hairpin RNA (shRNA)-mediated silencing of genes encoding PI3K family members. The data presented here supports the widely accepted idea that individual PI3K isoforms contribute differentially to cellular responses and provides further insight into the importance of this pathway in the regulation of a variety of processes, ranging from proliferation to migration.

The main focus was on PI3K/Akt signaling in NB, a pediatric cancer which derives from precursor cells of the neural crest (68). This pathway has been shown to be crucial for the proper regulation of cellular responses in NB. It is activated in response to growth factor stimulation (295) and contributes to chemosensitivity (266). Furthermore, a recent study demonstrated a correlation between activation of Akt and poor prognosis in NB patients (248). While mutation of *PIK3CA* and loss of *PTEN* are frequently detected in various human cancers, these genetic alterations do not seem to be the main contributors to aberrant PI3K/Akt signaling in NB (198, 216). Therefore, the mechanisms leading to pathway activation remain to be elucidated. We could show for the first time that the class I_A PI3K isoform p110δ plays an important role in NB growth and survival. This isoform was found to be over-expressed both at the mRNA and protein level in a subset of primary tumors and cell lines. Interestingly, increased expression of p85α/p110δ was mainly detected in children under the age of one and was found to inversely correlate with *MYCN* amplification. Considering that *PIK3CD* lies at 1p36.2, it is not surprising that expression of this gene was significantly lower in NB samples with 1p36 LOH (36). It was recently shown that decreased expression of *PIK3CD* correlates with poor clinical outcome (37). Moreover, it was observed that 1p36 LOH is associated with *MYCN* amplification (22). Therefore, it is conceivable that reduced expression of *PIK3CD* correlates with adverse outcome due to its association with numerous markers of poor clinical prognosis, including age at diagnosis (>1 year), 1p36 LOH

or *MYCN* amplification. An interesting observation is the inverse correlation between *PIK3CD* expression and *MYCN* amplification. A number of recent studies have reported interactions between the PI3K/Akt pathway and Myc-family proteins. It was shown that c-Myc expression had a negative impact on both basal and growth factor-induced activation of PI3K/Akt signaling (296). Moreover, it was observed that treatment of *MYCN*-amplified NB cell lines with LY294002 (a broad-spectrum PI3K inhibitor) resulted in decreased N-Myc protein levels, while mRNA levels remain unaltered (269). A further study showed that treatment of NB cells with rapamycin (an mTOR inhibitor) led to reduced protein expression levels of N-Myc (Oncogene, in press). It is therefore conceivable that *MYCN*-amplified NB display reduced *PIK3CD* expression due to one of the following mechanisms: (i) 1p36 LOH, (ii) a direct negative effect of *MYCN* amplification on *PIK3CD* expression or (iii) because they are less dependent on p85α/p110δ signaling. Interestingly, a recent study suggested that expression of *PIK3CD* is regulated by an epigenetic mechanism, and put *PIK3CD* forward as one of the most attractive genes for further studies of NB development and progression (213).

Of the three class I_A PI3K isoforms, p110δ has been found to show the most tissue-specific expression pattern in normal tissues. Expression of p110δ is predominantly limited to cells of the hematopoeitic system and it is therefore not surprising that increased expression has been observed in blast cells of AML patients (297). However, high levels of p110δ have also been detected in other human cancer cell lines (148, 298), indicating that aberrant expression of this isoform may contribute to the malignant properties of cancer cells. In support of this notion, the ability of p110δ to induce the transformation of chicken fibroblasts was recently demonstrated (299). Moreover, this isoform has been shown to contribute to migration in breast cancer cell lines (148) and was found to play a major role in regulating cell proliferation, chemoresistance and activation of Akt in AML cells (294, 297). The observation that p110δ is over-expressed in a subgroup of NB patients and contributes to the survival and proliferation of these cells is therefore a novel finding. We could further show that the class I_A PI3K isoforms p110α and p110δ do not have overlapping functions in NB cell responses. In SH-SY5Y cells, which express high levels of both isoforms, p110δ appeared to play a major role in controlling cell growth and survival under limiting growth conditions via the mTOR/S6K pathway. In contrast, in LA-N-1 cells which express only very low levels of p110δ, downregulation of p110α had a more prominent effect on cell growth and pathway activation. Interestingly, downregulation of p110α or p110δ did not affect Akt

activation in response to stimulation with EGF or IGF-I, while mTOR/S6K signaling was impaired. This finding suggests that activation of Akt and mTOR/S6K have different sensitivities to downregulation of class I_A PI3Ks. Furthermore, it is conceivable that other PI3K family members can compensate for p110α or p110δ in growth factor-stimulated Akt activation (298). In addition, we found decreased levels of the anti-apoptotic Bcl-2 protein in SH-SY5Y cells with low levels of p110δ. Based on the observation that Bcl-2 levels were elevated in SH-SY5Y cells maintained in high serum conditions, it can be assumed that growth factors present in the serum contribute to the regulation of Bcl-2 family member expression in these cells. Indeed, it has been shown that IGF-I, which is present in FCS, is involved in regulating levels of Bcl-2 (300). In view of the fact that p110δ contributes to IGF-I signaling in NB cells and that downregulation of p110α had a less pronounced effect on proliferation under low serum conditions, it can thus be postulated that p110δ is selectively involved in regulating the levels of Bcl-2 family members. However, further studies are necessary to validate this hypothesis. From a clinical point of view, these findings indicate that the class I_A PI3K isoform p110δ may play a role in the development of NB in very young patients (<1 year) or in a subset of tumors which do not harbor 1p36 LOH and/or *MYCN* amplification. From a mechanistic point of view, the results presented here are consistent with the idea that individual PI3K family members contribute differentially to biological responses and thus have non-redundant functions.

Another project aimed at elucidating the role of PI3K family members in GB also revealed specific roles for individual isoforms. A variety of biological responses were evaluated following treatment of cells with pharmacological isoform-specific PI3K inhibitors or upon downregulation of individual isoforms with siRNA. In addition to human cell lines, primary human *ex vivo* GB cultures were analyzed, thus providing a more detailed insight into the underlying mechanisms of GB biology. Our preliminary findings suggest that the class I_A PI3K isoforms p110α and p110β are involved in regulating various biological processes crucial for GB survival. Treatment of T98G or U251 cells with an inhibitor of p110α led to decreased basal activation of Akt and S6K. This finding is in line with a report involving the dual p110α/mTOR inhibitor PI-103, where a dose-dependent abrogation of basal Akt activation was observed in glioma cells (289). However, a subsequent report demonstrated that PI-103 also has comparable activity against the other class I catalytic PI3K isoforms

(290), thus explaining the efficient inhibitory effect of this compound on basal phosphorylation of Akt. Nevertheless, these results show that targeted inhibition of PI3Ks can abrogate constitutive PI3K/Akt pathway activation. This is of particular interest, considering that genetic alterations involving *PTEN* are frequent events in GB and are associated with elevated Akt activity (97). Moreover, basal PI3K/Akt pathway activation has been shown to modulate the response to chemotherapeutic agents, such as EGFR inhibitors (271, 272). PI-103 also showed very promising results *in vivo* (289). In xenograft models, mice treated with the compound showed markedly reduced tumor size compared to control animals, while no signs of toxicity were observed (289). Analysis of tumor tissue from treated animals revealed decreased levels of phosphorylated Akt and S6 protein, thus confirming the results obtained *in vitro* (289). Our further studies showed that p110α has an influence on the ability of GB cells to initiate PI3K pathway activation in response to growth factor stimulation. This is in line with the finding that inhibition of p110α has a negative effect on Akt activation. Interestingly, we could also show that co-treatment of GB cell lines and *ex vivo* cultures with a p110α inhibitor sensitized cells to treatment with chemotherapeutic agents. Similar findings have been reported upon co-treatment of cancer cells with LY294002 or Wortmannin (261, 281, 282). In additional studies, we found that p110β seems to contribute specifically to the regulation of cell motility and anchorage-independent growth. Interestingly, a recent study also reported a specific effect of the p110β inhibitor TGX-221 on anchorage-independent growth in chicken embryo fibroblasts (301). This is consistent with an earlier report, in which it was shown that a constitutively active p110β construct induced colony formation in human mammary epithelial cells plated in soft agar (302). We plan to further analyze the mechanisms underlying these observations.

Additional studies aimed at assessing the role of the less investigated class II isoform PI3KC2β revealed that this family member contributes to the regulation of various biological responses in NB and GB. Treatment of NB and GB cell lines with increasing concentrations of a pharmacological inhibitor of PI3KC2β (PI701) led to a dose-dependent inhibition of proliferation with IC_{50} values in the low micromolar range. Expression analysis revealed increased levels of this isoform in a subset of NB cell lines as well as in NB patients under the age of one, when compared to normal adrenal gland. In GB, subsets of cell lines and *ex vivo* cultures also displayed increased expression of PI3KC2β when compared to normal human

brain or cerebellum tissue. These findings are in line with previous reports where over-expression of this isoform was detected in a subset of tumors and cell lines from AML, GB and SCLC (154, 202, 298). Co-treatment of GB cell lines and *ex vivo* cultures with PI701 sensitized cells to Doxorubicin and led to increased cleavage of caspase 3 when compared to single-agent treatment. Additional studies in other cancer cells including medulloblastoma, AML and SCLC confirmed the contribution of PI3KC2β to cellular responses, such as basal proliferation, chemosensitivity or migration. Together, our studies showed that PI3KC2β is over-expressed in a broad spectrum of human cancer cells and could prove to be an attractive target for future cancer treatment, considering that pharmacological inhibition of this isoform not only reduced basal cell proliferation but also had an effect on chemosensitivity and cellular migration.

The PI3K pathway is frequently affected by a variety of genetic alterations (188) ranging from loss of tumor suppressor function (105) to point mutation or amplification of signaling molecules (191, 194, 208, 211, 215, 301). A number of these chromosomal abnormalities have been shown to contribute to aberrant pathway activation (97) or to confer oncogenic potential to the affected proteins (196, 240, 299). Reports concerning the patterns of these alterations are conflicting. While mutations in *PIK3CA* and *PTEN* seem to be mutually exclusive in GB (200-203), gastric carcinoma (303) and large B-cell lymphoma (304), the co-existence of *PIK3CA/PTEN* mutations has been reported in endometrial carcinoma (305) and breast cancer (306). Interestingly, some genes seem to be targeted by multiple mechanisms, such as *PIK3CA*, which encodes the catalytic class I_A PI3K p110α. A number of studies have reported point mutations in *PIK3CA* in different human cancers (192-194), which have been shown to have oncogenic potential *in vitro* (195-197). Amplification of *PIK3CA* has also been detected in numerous human malignancies (205-207) and could represent an alternative mechanism of pathway activation in the absence of mutations. This idea is substantiated by the observation that amplification of *PIK3CA* is a rare event in breast cancer, where mutation of this gene is one of the most frequent genetic alterations detected (208, 209). Although is well established that mutation of *PIK3CA* leads to activation of Akt *in vitro* (195, 196, 307) and it has been shown that tumor samples harboring *PIK3CA* mutations frequently display elevated levels of phosphorylated Akt (209, 308), the role of gene amplification is less clear. *In vitro* studies have demonstrated that over-expression of wild-type p110α is not sufficient to induce PI3K/Akt pathway activation or oncogenic

transformation (299). Analysis of primary tumor samples with *PIK3CA* amplification has demonstrated that this genetic alteration correlates with increased Akt activation in some cancers (205, 309, 310), while an effect on downstream signaling is not apparent in other cancers (311). Interestingly, over-expression of p110δ was found to lead to increased activation of the PI3K/Akt pathway under serum starved conditions and to induce cellular transformation in chicken embryo fibroblasts (299, 301). The observed effects were comparable to those exerted by the H1047R mutant of p110α (299), demonstrating that over-expression of p110δ could potentially contribute to the tumorigenic properties of cancer cells. The effect of p110δ on Akt/mTOR/S6K pathway activation was also observed in our studies involving NB (Paper 3.2.1), where shRNA-mediated downregulation of this isoform led to decreased levels of basal S6 protein phosphorylation, as well as impaired pathway activation upon stimulation with EGF or IGF-I. The effects were comparable with those observed upon downregulation of p110α, indicating that p110δ contributes substantially to PI3K/Akt/mTOR/S6K pathway activation in NB cells. Considering that components of this pathway are subject both to genetic mutation and amplification, it remains to be investigated which alteration represents a more beneficial target. Both of these chromosomal aberrations can lead to increased sensitivity to targeted therapies, as exemplified by the EGFR. Over-expression of the EGFR has been shown to correlate with increased sensitivity to EGFR inhibitors in certain cancers (271, 312). In addition, expression of the truncated form EGFRvIII has been found to predict sensitivity to EGFR inhibitors (272, 313). As with the EGFR, it could well be that both over-expressed and mutated p110α render a cancer cell more susceptible to targeted therapies. In this case, a detailed analysis of tumor samples will be crucial to identify patients most likely to benefit from specific PI3K inhibition.

Childhood and adult cancers differ in their pathogenesis. Cancer is thought to be a multi-step process ultimately leading to the progressive transformation of normal human cells (314). Numerous environmental factors are known to induce genetic alterations, such as exposure to UV irradiation, tobacco carcinogens or even chronic infections by the hepatitis B virus (315). It is therefore not surprising that the cancer incidence in a population increases with age (314). However, it can be assumed that the majority of these environmental influences are not involved in the development of pediatric cancers. In view of the fact that only 1-10% of all childhood malignancies arise from a predisposition (316), the events

leading to malignant transformation of pediatric cells remain largely unclear. While a number of mechanisms can contribute to the tumorigenic properties of cells in adults, only a few of these alterations have been detected in pediatric cancers (317). These include increased gene expression, fusion events leading to the generation of chimeric transcription factors or proteins with altered kinase activity as well as deletion of tumor suppressor genes (317). Indeed, comparison of the mechanisms underlying glioma formation in adults and children has revealed that some of the most prominent alterations found in adult gliomas, such as loss or mutation of *PTEN* or *EGFR* amplification are not detected in pediatric tumors (217, 318).

In GB, components of the PI3K pathway are affected by both amplification and mutation (discussed in Chapter 2.5). In NB, characteristic chromosomal abnormalities of genes encoding PI3K signaling proteins, such as mutation/amplification of *PIK3CA* or loss of PTEN function, are largely absent (198, 210, 216). Nevertheless, basal activation of Akt is detected in NB samples and has been shown to predict poor clinical outcome (248). Upon comparison of the alterations affecting genes involved in PI3K signaling in GB and NB, it becomes increasingly clear that despite many differences, aberrant PI3K/Akt pathway activation is a common feature in these human malignancies and contributes substantially to cellular responses such as proliferation or chemosensitivity (96, 247, 248, 266, 271). Along with numerous other studies reporting aberrant PI3K/Akt pathway signaling in a variety of human cancers (95, 195, 205, 239, 297, 319), the central role of this pathway in tumorigenesis is evident.

In summary, this thesis provides an extensive overview of the role of PI3K signaling in NB and in GB. It highlights the functional differences of individual catalytic PI3K isoforms, thus confirming the idea that the members of this family of proteins can have specific and non-overlapping functions in cellular responses. In particular, we could show a novel role for the class I_A PI3K isoform p110δ in regulating NB cell growth and survival. Our studies in GB revealed that the class I_A PI3K isoform p110α contributes to basal and growth factor-induced PI3K/Akt pathway activation, while p110β seems to be involved in regulating anchorage-independent growth and cellular motility. Finally, the class II PI3K isoform PI3KC2β was found to play a crucial role in modulating the sensitivity of GB cells to chemotherapeutic agents. The involvement of PI3K/Akt signaling in human malignancies has sparked interest in targeting PI3Ks as a novel therapeutic approach (188, 189, 275-277). Their tissue-specific distribution and non-redundant contributions to cellular responses make them particularly

attractive targets. While the use of small molecule PI3K inhibitors as first-line treatment is presumably not feasible, their application in combination with other agents, such as chemotherapeutics, could very well be beneficial for patients. Moreover, selective targeting of individual isoforms could circumvent the toxicity observed with broad-spectrum PI3K inhibitors, such as LY294002 or Wortmannin (277). A better understanding of the unique roles individual PI3Ks play in biological responses along with a new generation of isoform-specific PI3K inhibitors will hopefully allow the implementation of preclinical studies in clinical trials within the near future.

4.2 Future Perspectives

In recent years, there has been a growing interest in developing more specific therapeutic agents to combat cancer cells. New technologies, such as microarray analysis, have allowed large-scale comparison of the molecular fingerprints of different cancers, thereby generating huge amounts of information. One of the greatest challenges for the near future will be to critically review and validate the obtained results, in order to identify proteins that can be used as molecular markers and are realistic targets for inhibition by pharmacological compounds. A further challenge is posed by the development of truly specific kinase inhibitors, due to high sequence homology among members of certain protein families, such as the catalytic class I_A PI3Ks. Moreover, a detailed understanding of the inhibitory profile of novel compounds against other kinases will be indispensable to allow the use in clinical trials.

A number of groups have performed microarray analysis of NB tumors and cell lines in an attempt to gain a more detailed understanding of the molecular differences among patient groups. Interestingly, various independent screens have reported the differential expression of *PIK3CD*, the gene encoding the class I_A PI3K isoform p110δ, while the expression levels of other catalytic PI3K isoforms did not appear to differ in subsets of tumors. While this finding is certainly of great interest, a large amount of work remains to be done. We could show for the first time that this isoform plays a crucial role in regulating NB cell growth and survival through the mTOR/S6K pathway. However, the exact mechanism remains to be elucidated. Is there a direct link between p110δ and mTOR? How are the levels of phosphorylated Akt in response to growth factor stimulation maintained in the absence of p110α or p110δ? How is

p110δ involved in regulating the levels of the Bcl-2 family of proteins? It would certainly also be interesting to analyze the activation status of the mTOR/S6K pathway in primary tumors by means of a tissue microarray and see if there is a correlation with p110δ expression levels. Furthermore, it remains to be determined if a p110δ-specific pharmacological inhibitor would show the same effects observed in the cells stably expressing decreased levels of p110δ.

Our observations concerning the differential contribution of p110α, p110β and PI3KC2β to numerous biological responses in GB are interesting and support the idea that the various PI3K isoforms have non-overlapping functions. However, considering that most of the studies were performed with isoform-specific pharmacological inhibitors, it is absolutely necessary to validate these results with experiments based on downregulation of the different isoforms by means of siRNA. If the results can be confirmed, there are certainly a number of aspects that would be interesting to further investigate. Is the effect of p110α-inhibition on growth factor-induced PI3K/Akt activation reflected in a decreased proliferative response? How do p110α and PI3KC2β contribute to the observed effects on chemosensitivity? And further, how is p110β involved in regulating anchorage-independent growth and cellular motility?

In summary, there are quite a few issues that could be addressed based on the results presented in this thesis. While each new finding will certainly raise a number of new questions, the picture of PI3K signaling will hopefully become more complete in the near future, thus allowing the identification of promising new therapeutic targets for further investigation.

5 Review Articles

5.1 IGF-IR: Potential Role in Antitumor Agents

Drug News Perspect 19(5), June 2006

Available on the web at: www.prous.com/journals

LOOKING AHEAD

A wide variety of strategies are available to target IGF signaling, and the transition to a comprehensive evaluation in clinical trials can be expected in the near future.

IGF-IR: Potential Role in Antitumor Agents

by Ana S. Guerreiro, Danielle Boller, Kathrin T. Doepfner and Alexandre Arcaro

Summary

As insulin-like growth factor (IGF) signaling has been recognized to play an important role in human cancer, the IGF-I receptor (IGF-IR) is currently the focus of intensive research aimed at developing novel antitumor agents. The IGF system is frequently deregulated in cancer cells by the establishment of autocrine loops involving IGF-I or -II and/or IGF-IR over-expression. Moreover, epidemiological studies have suggested a link between elevated IGF levels and the development of major human malignancies, such as breast, colon, lung and prostate cancer. Experimental therapies aimed at inhibiting IGF signaling in human tumors involve various approaches, including neutralizing antibodies and pharmacological inhibitors of IGF-IR kinase activity. Although there are numerous reports describing the antitumor activity of such agents against human cancer cell lines propagated *in vitro* or in experimental animals, it remains unclear how soon the existing drugs will have a demonstrable effect in patients. In this review, we will discuss the evidence implicating the IGF signaling system in the pathology of human cancer and the various strategies that have so far been developed to target the IGF-IR.

The insulin/IGF family of cell signaling factors comprises a phylogenetically conserved system with a critical role in the growth and development of many tissues as well as the regulation of overall growth and metabolism. A high complexity is achieved by tight regulation of multiple proteins including three receptors (insulin receptor [IR], insulin-like growth factor I receptor [IGF-IR], IGF-II/mannose 6-phosphate receptor), three ligands (insulin, IGF-I and IGF-II) and six known types of circulating binding proteins (IGFBP1–6).[1–3] The IGF-IR is a transmembrane tyrosine kinase widely expressed in many human tissues and cell types with high homology to the insulin receptor (IR). However, experimental data suggest distinct roles for these two receptors, namely control of cell growth, differentiation and apoptosis by IGF-IR and the regulation of physiological processes by the IR.[4–6]

The IGF-IR is a heterotetrameric glycoprotein composed of two α and two β subunits, posttranscriptionally linked by disulfide bonds (α2β2). The α chain is extracellular, while the β chain is composed of a short extracellular region, a single transmembrane domain and a cytoplasmatic portion harboring the catalytic tyrosine kinase domain flanked by two regulatory regions and a juxtamembrane region that serves as a docking site for signaling molecules.[7] Activation of the receptor is achieved by binding of the specific ligand to the extracellular α subunit, triggering autophosphorylation of three tyrosine residues within the kinase domain of the β subunit.[8,9]

IGF-I and IGF-II are single-chain polypeptides with sequences 62% identical to pro-insulin. However, they are not proteolytically cleaved but remain linked in their mature form as four peptide domains.[10,11] Unlike other peptide hormones, the production and storage of these growth factors is not organ- or cell type-restrict-

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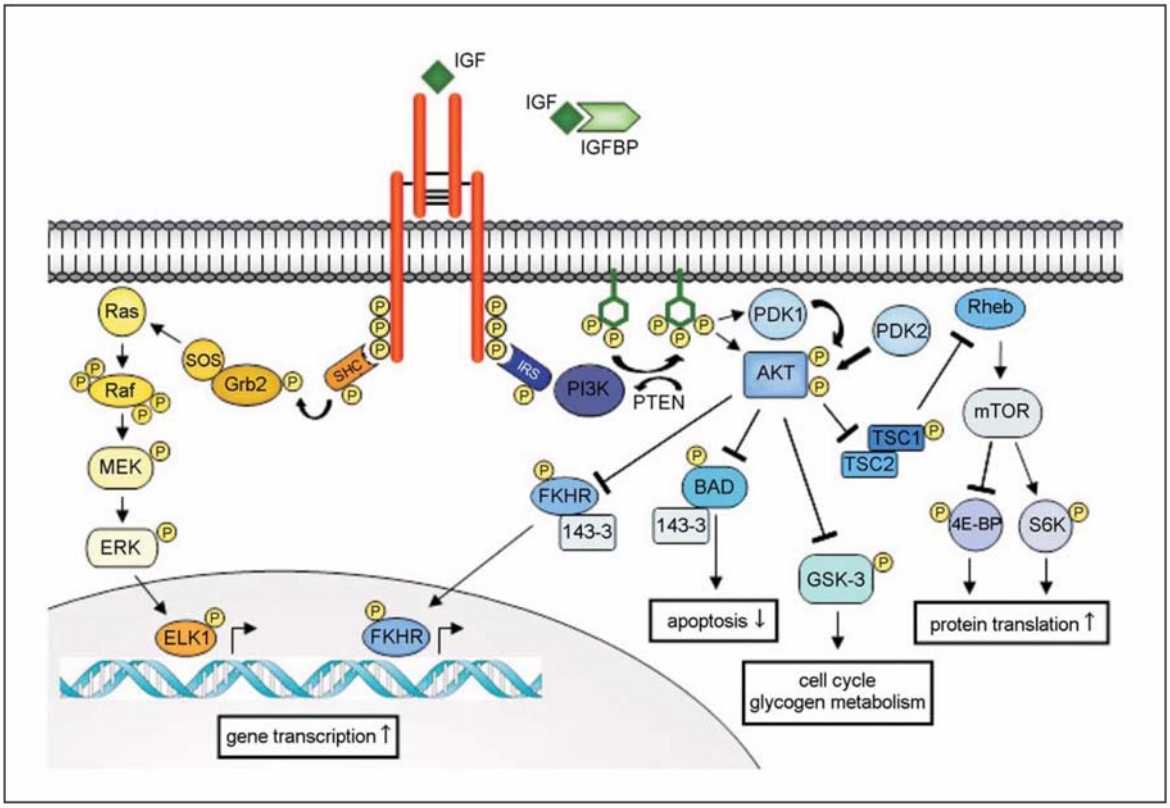

Fig. 1. Overview of the insulin-like growth factor I receptor (IGF-IR) signaling cascade. Binding of a specific ligand (IGFs) induces autophosphorylation and activation of the receptor, stimulating intracellular signaling networks. Key downstream pathways include the phosphoinositide 3-kinase (PI3K)/Akt cascade involving phosphoinositide-dependent kinases 1 and 2 (PDK1, PDK2), forkhead (FKHR), glycogen synthase kinase-3 (GSK-3), tuberous sclerosis complexes 1 and 2 (TSC1, TSC2), BAD, Ras homologue enriched in the brain (Rheb), the mammalian target of rapamycin (mTOR), 4E-binding protein (4E-BP) and ribosomal protein S6 kinase (S6K) as signal transducers and the Ras/Raf/extracellular signal-regulated kinase (ERK) cascade. Signaling by this system controls cell responses such as cell proliferation, growth and survival. Bioavailability of the ligand is partly regulated by IGF-binding proteins (IGFBPs).

ed, even though the main fraction originates from the liver. Instead, IGFs may be produced by almost any human cell.[12] The half-lives, transportation and bioavailability of IGFs circulating at high concentrations in the bloodstream and extracellular fluids are modulated by several high-affinity IGF-binding proteins (IGFBP1-6). More than 99% of the circulating IGFs are bound to IGFBPs or can be found in a ternary complex with IGFBPs and a third component, the acid-labile subunit (ALS).[13] The IGFBPs themselves are tightly regulated by tissue specificity, cell or matrix association, phosphorylation and proteolysis by various proteases.[14,15]

Both IGF-I and IGF-II interact with the IGF-IR, although IGF-I shows a much higher affinity than IGF-II. The closely related insulin, the main ligand for the IR, has an IGF-IR-binding affinity which is less than 1% of that of IGF-I.[16] The intrinsic receptor for IGF-II, the mannose-6-phosphate receptor (M-6-PR), differs significantly from the IGF-IR, possesses no tyrosine kinase activity and was reported to target IGF-II for lysosomal degradation without inducing a specific cell response.[17] The abilities of the highly homologous IGF-IR and IR to form hybrid receptors by dimerization of their hemi-receptors further increases the complexity of the signaling system. Such IGF-IR/IR hybrid receptors have been reported to influence cell responses by altering the affinities of their growth factor ligands.[18]

The activated autophosphorylated IGF-IR undergoes major conformational changes, providing docking sites for the recruitment of substrate proteins in order to initiate intracellular signaling cascades (Fig. 1). Thus far, the best-characterized signaling pathways involve the insulin receptor substrates-1 to -4 (IRS-1 to -4) and the Src-homology collagen protein (Shc) isoforms as adapter substrates. The adapter proteins bind to specific phosphotyrosine residues within the juxtamembrane region of the cytoplas-

matic IGF-IR and undergo subsequent tyrosine phosphorylation. Phosphorylation of the IRS adapter molecules on one-hand triggers activation of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway,[19–21] whereas, on the other hand, the Shc adapter activates signaling by the Ras/Raf/MAP-kinase pathway (reviewed in ref. 21). Generally, signals controlled by the IGF-IR have pleiotropic effects on the cell behavior controlling cell proliferation, differentiation and cell migration, but also regulating the apoptotic machinery.[22]

The role of the IGF-IR in human cancers

In recent years, a growing body of evidence has arisen, suggesting a key role for IGF signaling in various types of human cancers. Since the signals emanating from activated IGF-IR regulate cell proliferation, survival, differentiation and transformation, mechanistic studies have sought to define the role(s) of this receptor in the neoplastic phenotype. While the involvement of the IGF-1R in stimulating cell proliferation was an early finding, a number of studies performed in the last two decades have included experiments aimed at investigating the role of this receptor in the transformation of cells, as well as in metastatic events. Overexpression of IGF-IR in NIH 3T3 cells lead to a fully transformed phenotype, including anchorage-independent growth and loss of contact inhibition, as well as rapid tumor formation in nude mice.[23] More recently, a novel animal model was generated involving transgenic expression of a fusion receptor that is constitutively activated by homodimerization.[24] The fusion gene was placed under the control of the mouse mammary tumor virus promoter, which drives transcription in the mammary and salivary glands of transgenic mice. The incidence and kinetics of tumor emergence in these animals suggest that the transgene is sufficient to initiate and maintain the transformation process. Transfection of a cell line established from a salivary gland adenocarcinoma with small interfering RNAs against the IGF-IR resulted in a 50% reduction in thymidine incorporation, emphasizing the effect of deregulated signaling via the IGF-IR on cell proliferation.[24] Alternatively, other studies have investigated the effect of decreased IGF-IR signaling. Mouse embryo fibroblasts (MEFs) homozygous for a targeted disruption of the Igfr1 gene showed slower growth than wild-type cells in serum-containing medium and failed to form colonies in soft agar, even when stably transfected with activated Ras, a crucial downstream signaling mediator.[25] Another approach to study impaired IGF-IR signaling was the generation of LCC6 cells, a metastatic variant of a breast cancer cell line, expressing a C-terminally truncated IGF-IR, which acts as a dominant-negative receptor.[26] These cells showed decreased activation of downstream mediators, such as IRS-1 and IRS-2, as well as Akt. While proliferative responses of these cells to IGF-I or serum were not affected *in vitro*, they showed decreased anchorage-independent growth. Furthermore, analysis of xenograft tumor growth revealed that in contrast to wild-type cells, cells expressing the dominant-negative IGF-IR did not metastasize to the lungs, although they were equally aggressive locally when compared to wild-type cells.[26]

While no cancer-specific mutations of the IGF-IR or its ligands have been described to date, numerous studies have provided evidence for a link between this signaling pathway and the risk of developing cancer (reviewed in ref. 27). The most common findings associated with deregulated IGF signaling are overexpression of the IGF-IR, or the establishment of autocrine or paracrine signaling loops (Table I). While high expression levels of IGF-IR have been found in breast and colorectal cancer,[28,29] autocrine signaling loops are a more common phenomena and have been reported in a wide variety of human malignancies.[30–32] Paracrine signaling has mainly been described for breast cancer, where stromal cells have been shown to produce IGF-I and IGF-II.[33,34] Population studies have further highlighted the importance of IGF signaling in some of the most common cancers (Table II). The most readily accessible components of the IGF signaling pathway are IGF-I, IGF-II and the IGFBPs, as their circulating concentrations can easily be determined from blood samples. It is important to mention that circulating IGF-I levels vary substantially between normal individuals.[35] Nevertheless, evidence from epidemiological studies has revealed a correlation between elevated IGF-I levels and an increased risk of cancer diagnosis (reviewed in ref. 36). Although the population studies did not always come to the same conclusions, a recent systematic review of these results led to the interpretation that circulating IGF-I levels are indeed related to a risk of several common cancers.[37] The most significant correlation between increased levels of IGF-I and the risk of cancer diagnosis was found for prostate cancer and colorectal cancer.[38–45] The majority of the studies observed that individuals with elevated levels of IGF-I had an increased risk of developing cancer. It is important to emphasize that the increases detected were modest, and therefore were not always confirmed in population studies, especially if cohort sizes were small.[46,47] In the case of breast cancer, elevated levels of IGF-I have only been associated with an increased risk for premenopausal women, while no association between circulating levels and cancer risk was found for postmenopausal women.[48–51] Although elevated levels of IGF-I were detected in patients newly diagnosed with lung cancer,[52] population studies were not able to confirm a direct link between plasma levels and cancer risk.[53–55] A possible explanation for this inconsistency is the strong influence of carcinogen exposure (e.g., cigarette smoking or asbestos) on the development of lung cancer. Although only the most common human cancers are discussed here, recent population studies have been extended to other

TABLE I. OVERVIEW OF EXPERIMENTAL FINDINGS SUGGESTING THAT AUTOCRINE OR PARACRINE SIGNALING MEDIATED BY IGF-II, AS WELL AS OVEREXPRESSION OF THE IGF-IR, PLAY AN IMPORTANT ROLE IN A VARIETY OF COMMON HUMAN CANCERS

IGF-I	
Central nervous system	Increased IGF-I gene expression when compared with normal brain tissue[101]
	IGF-I detected in cyst fluids of CNS tumors[102]
Pulmonary tissue	Elevated levels of IGF-I in human neoplastic tissue when compared with normal lung tissue[103]
	IGF-I precursor molecule detected in conditioned culture medium of two lung cancer cell lines[31]
Gastrointestinal system	IGF-I detected in conditioned culture medium of human pancreatic cancer cells[32]
Female reproductive system	IGF-I detected in breast cancer tissue, but not in breast cancer cell lines[33,34]
	IGF-I only detected in stromal cells adjacent to normal breast epithelial tissue[104]
	Increased IGF-I levels in cyst fluid from invasive malignant ovarian tissue compared with benign neoplasms[105]
Male reproductive system	IGF-I secreted by various prostate cancer cell lines[75]
IGF-II	
Central nervous system	Increased IGF-II gene expression when compared with normal brain tissue[101]
	Enhanced expression of IGF-II in meningiomas compared with normal brain tissue[106]
Gastrointestinal system	Increased IGF-II expression in malignant tissue when compared with normal adjacent tissue[62]
	Increased IGF-II expression in adenocarcinoma of the colon when compared with normal adjacent tissue[107]
	Increased IGF-II mRNA in colon carcinoma when compared with normal colonic mucosa[108]
	IGF-II expression detected in a panel of six human colon cancer cell lines[30]
Female reproductive system	IGF-II detected in breast cancer tissue, but not in breast cancer cell lines[34]
	IGF-II detected in the malignant epithelial cells and their adjacent stromal cells[104]
	IGF-II mRNA transcripts detected in cervical cancer cell lines as well as in primary cervical tumor cell cultures[109]
	IGF-II detected in conditioned medium of an endometrial cancer cell line[110]
Male reproductive system	IGF-II secreted by human prostate carcinoma cell line PC-3[111]
IGF-IR	
Central nervous system	Detection of functional IGF-IR in glial tumors; increased number compared to normal brain tissue[112]
Gastrointestinal system	Increased expression of the IGF-IR in adenocarcinoma of the colon when compared to normal adjacent tissue[107]
	Strong expression of the IGF-IR in nearly all colorectal cases investigated[29]
Female reproductive system	High expression of the IGF-IR in a large panel of breast cancer specimens[28]
	Increased IGF-IR expression detected in cervical cancer cell lines and primary cervical cancer cell cultures compared to normal ectocervical cells[109]

types of cancer and should be mentioned as well.[56–58]

Growth hormone (GH), which is produced in the pituitary gland, stimulates production of IGF-I. In patients with acromegaly, in which GH levels are elevated, retrospective studies suggest that GH hypersecretion modifies the progression of existing malignancies, particularly colon cancer.[59,60] A study performed on GH-treated cancer survivors found no increased risk of disease recurrence or death in these patients. While the overall incidence of secondary malignancies did not increase, survivors of acute leukemia and lymphoma had an elevated number of secondary solid malignancies.[61] The relatively marginal effect of excess GH/IGF on tumor onset and progression might indicate that the auto- and paracrine components of the mechanism are more important than the endocrine.

In summary, both mechanistic as well as epidemiological studies have provided valuable information concerning the role of IGF signaling in human cancers. Since this signaling pathway regulates essential processes such as cell proliferation, survival or differentiation, it might provide targets for the development of promising new therapeutic approaches, which could possibly be combined with other classical treatment regimens.

Strategies to target IGF-IR in cancer therapy

As clarification of the IGF pathophysiology in cancer has progressed, interest in the IGF-IR as a target in cancer treatment has increased. IGF-IR fulfills several criteria for an ideal pharmaceutical target of cancer chemotherapy: (i) it plays a crucial role in malignant transformation and tumor progression; (ii) components of the IGF system are differentially expressed in tumors compared with normal tissues; (iii) *in vitro* studies have clarified molecular mechanisms of IGF-IR activity and (iv) the receptor and its ligands are easily measurable in clinical samples.[62] Furthermore, various therapeutic strategies that target IGF-IR have demonstrated remarkable antitumor activity in tissue cultures and in mouse models of cancer (Fig. 2). Work is underway to produce clinically useful anticancer drugs, but so far it remains unclear whether preclinical studies will be successfully translated into clinical

TABLE II. OVERVIEW OF POPULATION STUDIES AIMED AT INVESTIGATING THE LINK BETWEEN CIRCULATING LEVELS OF SIGNALING COMPONENTS, SUCH AS IGF-I, IGF-II OR IGFBP-3 AND THE RISK OF DEVELOPING COMMON HUMAN CANCERS

STUDY	PARAMETER	CONCLUSION
Breast cancer	IGF-I; IGFBP-3	Association between high plasma levels of IGF-I and increased risk for breast cancer in premenopausal women[48]
	IGF-I; IGFBP-3; C-peptide	Association between high serum levels of IGF-I and increased risk for breast cancer in premenopausal women[49]
	IGF-I; IGF-II; IGFBP-3	High plasma levels of IGF-I and IGFBP-3 in patients with breast cancer. However, no significant association found for IGF-II[113]
	IGF-I; IGFBP-1, -2, -3; insulin; glucose	Association between high serum levels of IGF-I and IGFBP-3 and increased risk for breast cancer in premenopausal women[50]
	IGF-I; IGFBP-1, -2, -3; insulin	No association between plasma levels IGF-I and breast cancer risk in premenopausal women[114]
	IGF-I; IGFBP-1, -2, -3; C-peptide	No association between plasma levels of IGF-I, IGFBP-1, -2, -3 or C-peptide and breast cancer risk in postmenopausal women[51]
Prostate cancer	IGF-I; IGF-II; IGFBP-3	Association between high plasma levels of IGF-I and increased risk for prostate cancer[115]
	IGF-I; IGFBP-3	High serum levels of IGF-I in patients newly diagnosed with prostate cancer. No association between IGFBP-3 levels and cancer diagnosis[38]
	IGF-I; IGF-II; IGFBP-3; PSA	Association between high serum levels of IGF-I and low levels of IGF-II and risk for prostate cancer[41]
	IGF-I; IGFBP-1, -2, -3; insulin	Association between high plasma levels of IGF-I and IGFBP-3 and increased risk for prostate cancer[39]
	IGF-I; IGFBP-3	No association between serum levels of IGF-I or IGFBP-3 and risk for prostate cancer[46]
	IGF-I; IGFBP-3	Association between high plasma levels of IGF-I and low levels of IGFBP-3 and risk of developing advanced-stage prostate cancer[40]
	IGF-I; IGFBP-3	No association between serum levels of IGF-I or IGFBP-3 and risk for prostate cancer in smokers[116]
Lung cancer	IGF-I; IGF-II; IGFBP-3	High plasma levels of IGF-I and low levels of IGFBP-3 in patients with newly diagnosed lung cancer[52]
	IGF-I; IGFBP-1, -2, -3; insulin; cotinine	No association between serum levels of IGF-I or any of the investigated IGFBPs and lung cancer risk in women[53]
	IGF-I; IGFBP-3	No association between serum levels of IGF-I and lung cancer risk in men. Reduced lung cancer risk in individuals with high IGFBP-3 serum levels[54]
	IGF-I; IGFBP-3	No association between serum levels of IGF-I or IGFBP-3 and risk for lung cancer[55]
Colorectal cancer	IGF-I; IGF-II; IGFBP-3	Association between high plasma levels of IGF-I and low levels of IGFBP-3 and risk for colorectal cancer[42]
	IGF-I; IGFBP-3	No association between serum levels of IGF-I or IGFBP-3 and risk for colorectal cancer[47]
	IGF-I; IGFBP-3	Association between high plasma levels of IGF-I and low levels of IGFBP-3 and increased risk for colorectal cancer[43]
	IGF-I; IGFBP-1, -2, -3; C-peptide	Modest association between elevated serum levels of IGF-I and significant association between IGFBP-3 levels and risk for colorectal cancer[44]
	IGF-I; IGF-II; IGFBP-3	No association between IGF-I levels and risk of colorectal cancer. Significant positive association between serum levels of IGF-II and IGFBP-3 and risk for colorectal cancer[117]
	IGF-I; IGFBP-3	Association between high serum levels of IGF-I and risk for colon, but not rectal cancer[45]

PSA, prostate-specific antigen.

evaluation trials. The major challenge lies in designing strategies that specifically target the IGF-IR without interfering with the function of the closely related IR, producing secondary diabetogenic effects. Moreover, it is important not only to evaluate single agent activity, but also to consider the potential application of IGF-IR targeting strategies in combination with current cytotoxic drugs, radiotherapy and/or novel anticancer agents.

IGF-IR can be pharmacologically inhibited through multiple mechanisms: (i) reduction of IGF-I/IGF-II levels; (ii) inhibition of ligand binding; (iii) downregulation of IGF-IR expression; (iv) inhibition of IGF-IR

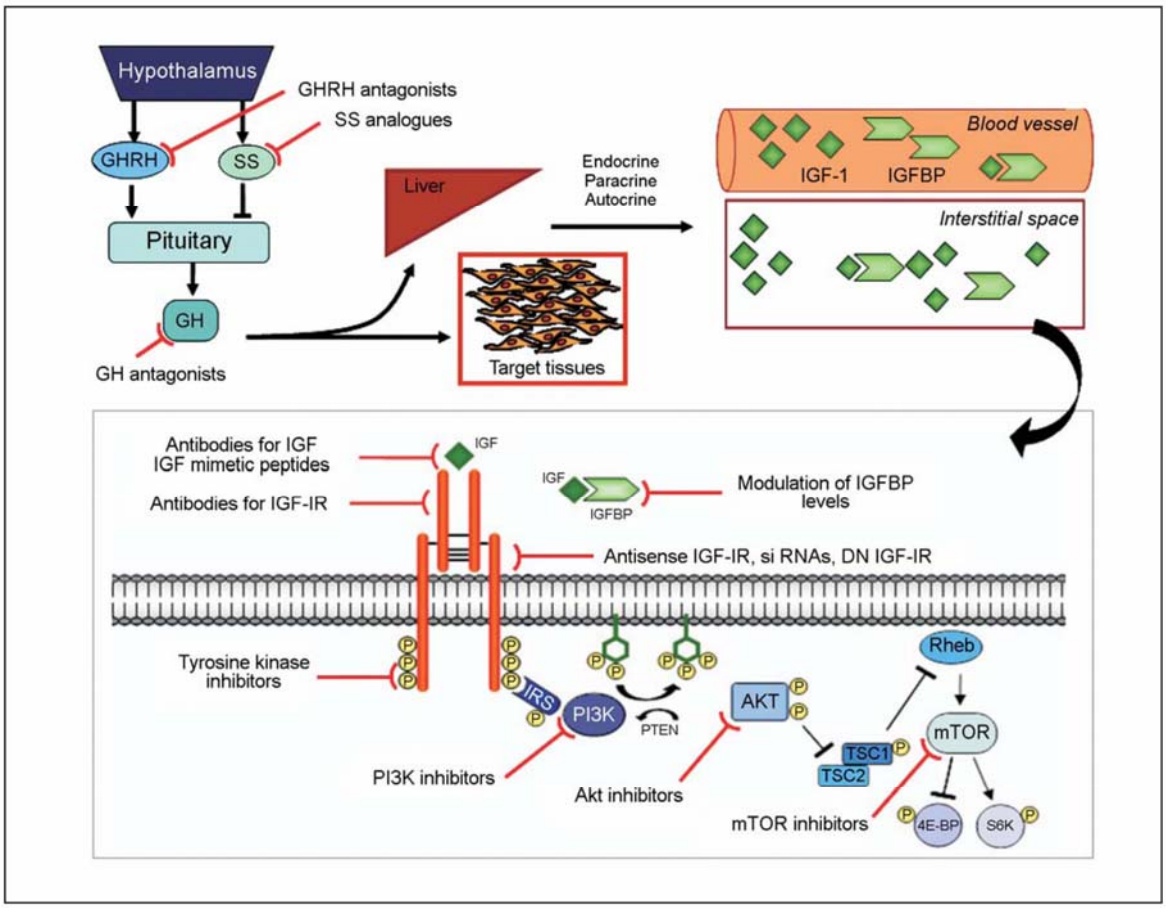

Fig. 2. Insulin-like growth factor system: targeting strategies. Growth hormone (GH), produced in the pituitary gland under the control of the hypothalamic factors growth hormone-releasing hormone (GHRH) and somatostatin (SS), stimulates production of IGF-I. Most insulin-like growth factors and insulin-like growth factor binding proteins (IGFBP) are produced in the liver and delivered to target tissues through the circulation via an endocrine mechanism. However, IGFs and IGFBPs can also be produced locally through autocrine and paracrine mechanisms. The GH/IGF axis can be disrupted by antagonists of GH, antagonists of GHRH or agonists of SS, leading to decrease in the circulating levels of IGF. Antibodies for IGF, IGF-IR, antisense RNA, small interfering RNA or dominant-negative[35] are other approaches that have successfully targeted IGF signaling in preclinical studies. Targeting intracellular molecules downstream of the IGF-IR, such as Akt and the mammalian target of rapamycin (mTOR), is also of great therapeutic interest since they are key regulators of IGF-IR function.

function and (v) targeting downstream signaling molecules. Several of these approaches are summarized on Table III and discussed below.

Reduction of IGF-I/IGF-II levels

IGF-IR requires ligand stimulation for activation; it is not activated by overexpression alone, and no activating mutations in the IGF-IR gene have been reported to date. Therefore, a rational approach to inhibit the IGF-IR is to reduce the levels of its main ligands, IGF-I and IGF-II, either with ligand-specific neutralizing antibodies or by decreasing IGF production.

The mouse monoclonal antibody (mAb) KM1468, which specifically binds to human IGF-I and IGF-II but not to insulin, markedly and dose-dependently suppressed the development of new bone tumors and the progression of established bone tumors in nonobese diabetic/severe combined immunodeficient (SCID) mice implanted with human adult bone.[63] KM1468 also suppressed hepatic metastasis induced by intrasplenic injection of the human colorectal cancer cell line HT-29 by blocking both endocrine and paracrine supplies of IGFs.[64] Concerns regarding the safety of long-term treatment with KM1468 must still be addressed, since IGFs are essential for a wide variety of cell types.

Recently, a novel fully human monoclonal, anti–IGF-II antibody m610, was characterized.[65] M610 bound with high (subnanomolar)

TABLE III. OVERVIEW OF IGF-IR TARGETING STRATEGIES

MECHANISMS OF INHIBITION	TARGETING STRATEGY
1. Reduction of IGF-I levels	a. Antibody to IGF-I/IGF-II[63–65]
	b. Reduction of growth hormone (GH)-induced production of IGF-I (somatostatin analogues, GHRH antagonists, GH antagonists)[66–68]
2. Blockade of ligand binding	a. Antibody to IGF-IR[69,70]
	b. IGF mimetic peptides[75]
	c. Inhibitory IGFBP[76]
3. Downregulation of IGF-IR expression	a. Antisense IGF-IR[78,118]
	b. Small interfering RNAs[79]
	c. Recombinant IGF-I/pseudomonas exotoxin fusion protein[119]
4. Inhibition of IGF-IR function	a. Small molecule IGF-IR tyrosine kinase inhibitors[85,90]
	b. Dominant negative IGF-IR mutants[94,95,120,121]
5. Targeting downstream signaling molecules	a. Akt inhibitors[98]
	b. Rapamycin/mTOR inhibitors[99]

affinity to IGF-II, did not cross-react with IGF-I or insulin and inhibited signal transduction mediated by IGF-IR/IGF-II interaction. It also inhibited growth of the prostate cancer cell line DU145 and inhibited migration of the MCF-7 breast cancer cell line *in vitro*.[65] It remains to be demonstrated whether m610 has the same inhibitory activities *in vivo*. One potential advantage of specifically targeting IGF-II, in contrast to IGF-I, is the fact that decreased concentrations of IGF-I trigger feedback upregulation by the human GH, whereas no such feedback mechanism has been associated with IGF-II. Furthermore, antibodies for IGF-I/II are not expected to interfere with insulin signaling.

Because serum IGF-I levels are controlled by GH, another strategy to remove the ligand would be the disruption of the growth hormone/IGF-I axis using somatostatin analogues,[66] growth hormone releasing hormone (GHRH) antagonists[67] or GH antagonists.[68]

Blockade of ligand binding

A number of monoclonal antibodies have been developed to target the receptor itself, which bind to the extracellular domains of the IGF-IR and block ligand binding.[69–72] The mouse monoclonal antibody EM164 potently inhibited the proliferation and survival of various human cancer cell lines *in vitro*, including breast, lung, colon, cervical, ovarian, pancreatic, melanoma, prostate, neuroblastoma, rhabdomyosarcoma and osteosarcoma cancer lines.[70] *In vivo*, EM164 caused regression of established BxPC-3 human pancreatic tumor xenografts in SCID mice.[70] The fully human anti–IGF-IR monoclonal antibodies A12[69] and CP-751,871[72] have also been characterized and showed strong antitumor activity *in vitro* and *in vivo*. The mAb CP-751,871 is currently in phase I clinical trials for multiple myeloma. A feature common to all anti–IGF-IR antibodies, probably more important than the blocking activity itself, is their ability to downregulate IGF-IR over time by promoting internalization of the receptor. However, even though none of the specific antibodies showed cross-reactivity with the insulin receptor, Sachdev et al. (2006) reported downregulation of IR by monoclonal antibodies against IGF-IR.[73] Lu et al. (2004) described a fully human bispecific antibody (BsAb) that blocked simultaneously the epidermal growth factor receptor (EGFR) and the IGF-IR. The BsAb effectively blocked not only the two ligands, EGF and IGF, from binding to their specific receptors, but also completely blocked the activation of Akt and MAPK induced by both EGF and IGF, thereby inhibiting tumor proliferation.[74] This BsAb format is an interesting approach for targeting simultaneously two tumor-associated molecules, enhancing antitumor activity without adding severe unwanted toxicities. Taken together, these findings suggest that receptor-targeting antibodies might have important therapeutic advantages, in the context of specificity and toxicity.

IGF mimetic peptides have been described and shown to compete with the binding of IGF-I to its receptor, inhibiting tumor growth.[75] However, their antitumor activity was never assessed *in vivo*.

IGF action is controlled by high affinity binding proteins. To date, six distinct IGFBP species have been cloned. Because IGFBPs have higher affinity for IGF-I and IGF-II than the receptors, it would also be possible to neutralize IGF action with these naturally occurring proteins.[76,77]

Downregulation of IGF-IR expression

Antisense strategies[78] and small interfering RNA[79] effectively inhibited tumor growth in laboratory studies, by reducing receptor expression.

Preclinical models showed inhibition of tumor growth and metastasis upon downregulation of IGF-IR using antisense strategies, including in malignant glioma.[80–82] Based on this knowledge, Andrews et al. (2001) treated 12 patients with recurrent malignant astrocytoma with antisense oligonucleotides directed against IGF-IR (phase I clinical trial).[83] Two complete responses and four partial responses were achieved.

Inhibition of IGF-IR function

As the first clinically successful tyrosine kinase inhibitor heralded the

triumph of targeted cancer therapy (imatinib mesylate, *Gleevec*), the development of pharmacological inhibitors targeting tumor-associated tyrosine kinases grew. In addition, several new compounds with enhanced specificity towards IGF-IR entered preclinical studies. Advances in the characterization of the structural biology of the insulin and IGF-I receptors were of great importance for the design of specific IGF-IR inhibitors.[84] García-Echeverría et al. (2004) characterized a small molecular weight kinase inhibitor of the IGF-IR, which was specific for IGF-IR at a cellular level, with *in vivo* and *in vitro* antitumor activity.[85] As a single agent, a similar compound, NVP-ADW742, showed significant antitumor activity in an orthotopic xenograft multiple myeloma model, providing further *in vivo* proof of principle for the use of selective IGF-IR inhibitors in cancer.[86] There are several lines of evidence suggesting that IGF-IR kinase inhibitors may well play an important role in combination therapies with established cytotoxic drugs. NVP-AEW541 sensitized Ewing's sarcoma cell lines to the chemotherapeutic agents vincristine, actinomycin D and ifosfamide, while combined treatment with NVP-AEW541 and vincristine significantly inhibited tumor growth of Ewing's sarcoma xenografts in nude mice.[87] Inhibition of IGF-IR signaling upon treatment with NVP-ADW742 synergistically enhanced the sensitivity of SCLC to etoposide and carboplatin, which are commonly used in the treatment of SCLC.[88] The same group had previously investigated the effect of NVP-ADW742 in combination with a c-Kit inhibitor. SCLC cell lines with an active SCF/c-Kit autocrine loop were resistant to a c-Kit inhibitor (imatinib mesylate) and NVP-ADW472. Interestingly, optimal growth inhibition was observed upon co-treatment with both imatinib mesylate and NVP-ADW472, suggesting that in tumors in which critical signal transduction pathways can be activated by alternative receptors, optimal therapy may require inhibition of multiple receptors.[89] Girnita et al. (2004) verified that the cyclolignan PPP inhibited the activity of IGF-IR without affecting the highly homologous insulin receptor or competing with ATP in an *in vitro* kinase assay, suggesting that it may inhibit IGF-IR autophosphorylation at the substrate level. In addition, PPP caused complete tumor regression in xenografted and allografted mice.[90] A general concern with antitumor agents is the acquisition of resistance in tumors. Vasilcanu et al. (2006) addressed this problem by investigating whether malignant cells may develop resistance to PPP. In a panel of 10 selected malignant cell lines with documented IGF-IR expression and apoptotic responsiveness to PPP treatment, no or remarkably weak resistance to the IGF-IR inhibitor PPP was observed over a period of 80 weeks.[91]

There are still no reports of receptor downregulation upon treatment with any pharmacological IGF-IR inhibitor. Moreover, during the first phase of an 80-week selection treatment with PPP, temporary and moderate increases in IGF-IR expression were observed, although such increases did not correlate with increased resistance to the drug.[91] In summary, IGF-IR kinase inhibitors are likely candidates to become anti–IGF-IR drugs. However, concerns regarding cross-reactivity with the insulin receptor and toxicity, as well as possible mechanisms of resistance, should be carefully addressed.

A wide variety of mutant IGF-IRs were developed and delivered to the cells, via transfection or infection, to test their dominant-negative potential in IGF-I sensitive cells. Various mutant receptors were designed, with deletions of different domains or by substitution of critical residues.[25,26,92–95] Despite positive experience with preclinical models, such approaches cannot be evaluated in clinical studies before optimizing general gene therapy protocols.

Targeting downstream signaling molecules

Targeting signaling pathways downstream of the IGF-IR is another approach to downregulate IGF signaling with significant therapeutic advantages. PI3K signaling is often activated in human cancers through a variety of mechanisms. Mutations in PTEN, the PI3Ks p110 (PIK3CA gene) and p85, as well as Akt appear with a combined frequency of 50% in human cancers.[96] Attractive downstream targets are PI3K,[97] Akt[98] and mTOR,[99] for which specific inhibitors have been developed and tested in preclinical studies. In a phase II clinical trial, the mTOR inhibitor and rapamycin analogue CCI-779 were not sufficiently active in melanoma to warrant further testing as a single agent,[100] suggesting that further efforts must be undertaken in order to understand rapamycin sensitivity and better identify potentially responsive patients.

Conclusion

Discovering targets for cancer treatment represents a challenge that goes far beyond identifying those molecules that play key roles in tumor development. Due to the complexity of the IGF system, many questions remain unanswered, and it is at present unclear how knowledge from preclinical studies may contribute to an improvement in cancer patient management. However, as summarized in this review, a wide variety of strategies are available to target IGF signaling, and the transition to a comprehensive evaluation in clinical trials can be expected in the near future.

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5.2 Recent Patents of Gene Sequences Relative to the Phosphatidylinositol 3-kinase / Akt Pathway and their Relevance to Drug Discovery

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Recent Patents of Gene Sequences Relative to the Phosphatidylinositol 3-kinase / Akt Pathway and their Relevance to Drug Discovery

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Abstract: Phosphoinositide 3-kinases (PI3Ks) play an essential role in the signal transduction events initiated by the binding of extracellular signals to their cell surface receptors. There are eight known PI3Ks in humans, which have been subdivided into three classes (I-III). The class I_A of PI3K comprises the p110α, p110β and p110δ isoforms, which associate with receptor tyrosine kinases (RTKs). On the other hand, the class I_B PI3K p110γ is regulated by G-protein-coupled receptors (GPCRs). Gene targeting studies in mice have revealed specific biological functions for the class I_A p110δ in lymphocyte activation, and the class I_B p110γ in inflammatory cell responses. In human cancer, recent reports have described activating mutations in the *PIK3CA* gene encoding p110α, and inactivating mutations in the *PTEN* gene, a tumor suppressor and antagonist of the PI3K pathway. Thus, individual PI3K isoforms are potential drug targets for a variety of human diseases, including allergies, cancer, rheumatoid arthritis and arterial thrombosis. In this review, we will discuss recent patents relating to class I PI3Ks, including patents on the cDNA sequences of p110γ and p110δ. Moreover, we will review patents on novel pharmacological PI3K inhibitors and on methods of manipulating T cell responses through PI3K.

Keywords: Phosphatidylinositol 3-kinase, Akt, receptor tyrosine kinase, G-protein-coupled receptor, cancer, inflammation, rheumatoid arthritis, allergy.

INTRODUCTION

PHOSPHOINOSITIDE 3-KINASES

The phosphoinositide 3-kinases (PI3K) are a family of evolutionary conserved lipid kinases, which play a crucial role in controlling a wide variety of intracellular signaling events. PI3Ks phosphorylate phosphatidylinositol (PI) on the D-3 position of the inositol ring, producing distinct second messengers such as PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 (PIP_3) [1]. These second messengers are known to activate diverse target proteins involved in complex signaling cascades, ultimately resulting in the activation of cellular responses including growth, proliferation, survival and motility.

The family of PI3Ks identified in various species can be subdivided into three main classes (class I-III), based on structural similarity and *in vitro* substrate specificity [1] (overview given in Fig. (**1**)). Class I_A includes p110α, p110β, and p110δ, which are known to form a heterodimeric complex with a p85, p55, or p50 (α or β) regulatory subunit. This adapter subunit contains two Src-homology 2 (SH2) domains mediating their association with activated tyrosine kinase-coupled growth factor receptors [1]. PIP_3 produced by class I_A PI3Ks activates the protein kinase phosphoinositide-dependent protein kinase-1 (PDK1), inducing the recruitment and activation of the key signal transducer protein kinase B (PKB)/Akt [2]. Akt is involved in regulating the cell cycle and glucose metabolism through glycogen synthase kinase-3 (GSK3) [3] and in modulation of cell growth and survival. Moreover, Akt controls the translational machinery through the mammalian target of rapamycin (mTOR), the ribosomal protein S6 kinase (S6K) and the 4E-binding protein (4E-BP) [4]. Downstream events controlled by Akt further include the control of apoptosis through the regulation of proteins such as forkhead (FKHR), BAD, NF-κB and murine double minute gene-2 (MDM-2) [5] (overview given in Fig. (**2**)). Expression studies of the class I_A PI3K isoforms have revealed a ubiquitous distribution of the subunits p110α and p110β in human tissues, whereas p110δ appears to be selectively expressed in leukocytes [6-8].

The class I_B of PI3Ks is composed of one enzyme only, p110γ, which functions through heterodimer formation with a regulatory subunit p101 [9] or p84 [10]. Activation of p110γ is controlled by receptors capable of activating heterotrimeric guanine nucleotide-binding proteins, termed G-protein coupled receptors (GPCRs) [9]. The PI3K p110γ is thought to link GPCR signaling to PIP_3 production, which controls cell motility in inflammatory cells such as macrophages and neutrophils [11] (Fig. **3**).

The human class II of PI3Ks comprises the three isoforms PI3KC2α, PI3KC2β and PI3KC2γ [1]. The hallmarks of class II family members are a substrate specificity restricted to PI and PI(4)P *in vitro* and a C-terminal C2 domain. Although the precise cellular function of these enzymes remains generally poorly understood, recent reports have described class II PI3Ks as downstream transducers of activated polypeptide growth factor receptors [12,13]. The class III of PI3K includes a homolog of the yeast vesicular

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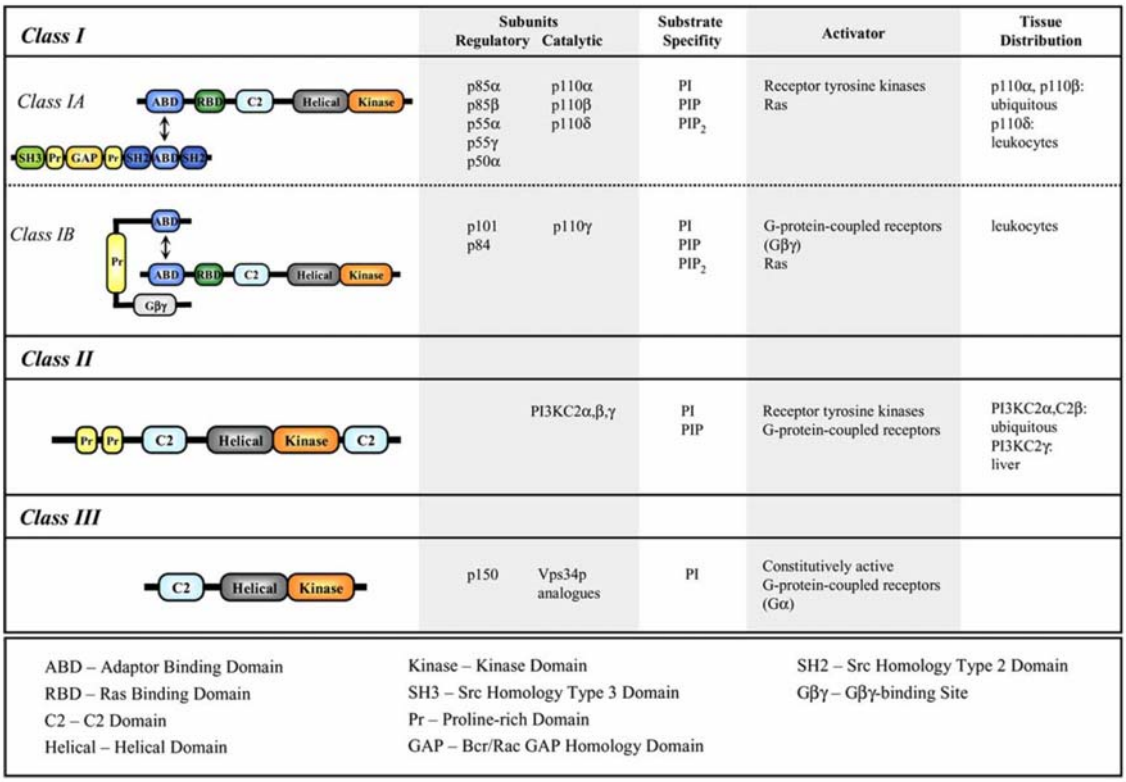

Fig. (1). Overview of PI3K isoforms. The PI3K family consists of eight isoforms, which are grouped into three classes, based on sequence homology and *in vitro* substrate specificity. The class I isoforms are further subdivided into class I_A and class I_B isoforms based on their mechanism of activation. While certain PI3K isoforms are expressed ubiquitously, isoforms such as p110γ or p110δ have been found to be more tissue-specific.

protein-sorting protein Vsp34 [14] and its major function is in intracellular trafficking events [15].

INHIBITION OF PI3K SIGNALING

As PI3Ks have been identified to play critical roles in distinct cellular signaling processes, a precise understanding of these kinases, their substrates and effectors is of particular interest. Anomalies in signaling cascades have been described in various human diseases and thus a better understanding of these events is of high importance in the search for therapeutic, diagnostic and screening applications. A number of attempts have been made to better understand PI3Ks in general, as well as to gain insight into the specific functions of the different isoforms. The first selective pharmacological PI3K inhibitors to have been described are wortmannin [16,17], a compound that was originally isolated from soil bacteria and is toxic to fungi, and LY294002 [18], a morpholino derivative of quercetin, a naturally occurring bioflavinoid and broad spectrum kinase inhibitor. Both compounds have been shown to inhibit cell growth at concentrations that would be expected to inhibit class I PI3Ks. However, as these pharmacological inhibitors display little selectivity within the PI3K family and might also affect other kinases, further research has been aimed at developing compounds with improved specificity and pharmacokinetic properties. Various PI3K inhibitor prodrugs and their possible pharmaceutical applications have been patented by Garlich *et al*. [19]. In addition, patents exist on various PI3K polypeptides, nucleic acid sequences encoding PI3Ks, antibodies that are specifically immunoreactive with PI3Ks and methods for using these reagents in screening and therapeutic applications.

GENE-TARGETING STRATEGIES

Gene-targeting strategies have been aimed at deleting specific PI3K isoforms and have uncovered key roles of the different enzymes in immunity, metabolism, cardiac function, cell proliferation and cancer susceptibility. Different knock-out mice with targeted deletions of genes encoding PI3K regulatory and catalytic subunits have been generated and phenotypically analyzed [20].

A knock-out mouse with a deletion in the gene of the PI3K class I_A catalytic subunit p110α has been generated by

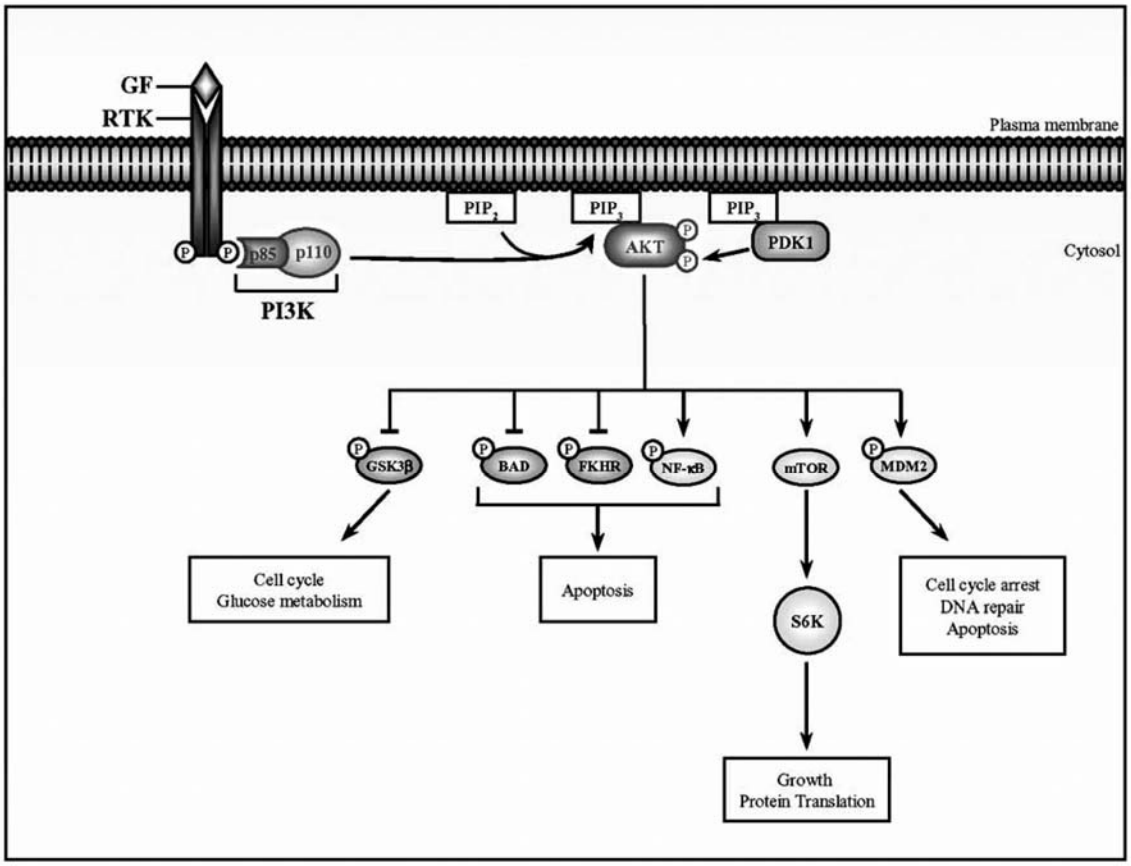

Fig. (2). Overview of the phosphoinositide 3-kinase (PI3K) signaling pathway. Upon growth factor (GF) binding, transmembrane receptor tyrosine kinases (RTK) are activated by autophosphorylation. PI3K activation occurs when the regulatory p85 subunit is recruited to specific phosphotyrosine sites located in the intracellular domain of the activated RTK. Key downstream signaling mediators include phosphoinositide-dependent kinase 1 (PDK1) which is essential for activation of protein kinase B (PKB/Akt). Effector molecules include glycogen synthase kinase-3 (GSK-3), BAD, forkhead (FKHR), nuclear factor kappa B (NF-κB), mammalian target of rapamycin (mTOR), ribosomal protein S6 kinase (S6K) and murine double minute gene 2 (MDM2).

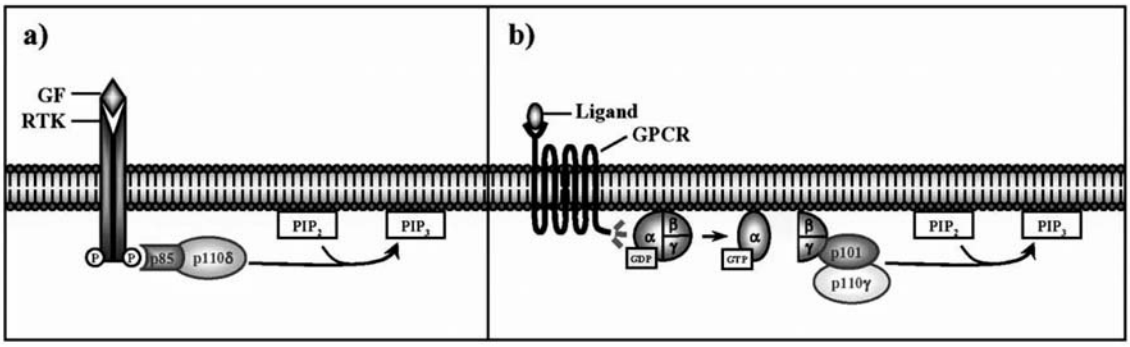

Fig. (3). Activation of p85/p110δ and p101/p110γ occurs via different receptors. While the p85/p110δ complex is recruited to activated receptor tyrosine kinases (RTK) following growth factor (GF) binding (a), the p101/p110γ complex is activated by the Gβγ subunit following binding of a specific ligand to G protein-coupled receptors (GPCR) (b).

targeting the p85-binding domain of the *PIK3CA* gene, leading to loss of expression of this specific isoform [21]. The $PIK3CA^{del/del}$ embryos showed a clear developmental delay and died between days 9.5 and 10.5 of embryonic development [21]. In embryos lacking the p110α enzyme, a profound proliferation defect could be observed, which was further supported by a failure in replication of p110α-deficient fibroblasts in culture medium, even when supplemented with growth factors. The developmental period between E9.5 and E10.5 is known for increased cellular proliferation, growth, and differentiation. It has thus been hypothesized that the intrauterine death of $PIK3CA^{del/del}$ embryos at this stage was caused by the inability to meet the increased demand in proliferative signals maintained by PI3K signaling through the p110α isoform [21]. Moreover, mice deficient in the p110α isoform displayed multiple vascular defects [22]. A role for p110α in the control of cell growth was further highlighted by an increase in heart size in transgenic mice expressing a constitutively active mutant of p110α [23]. Conversely, a decrease in heart size was observed upon expression of a dominant-negative mutant of this PI3K isoform in the heart [23]. The interpretation of knock-out data, however, has been complicated by the observation of an upregulation of other PI3K subunits after the deletion of one specific isoform. In $PIK3CA^{del/del}$ homozygous embryos there was an apparent increase in the expression of the PI3K class I_A regulatory subunit p85/p55, raising the question of a contributing phenotypical effect by these adapter proteins. Further insight into the function of p110α has been given by the generation of mice carrying a knock-in mutation (D933A) that abrogates the p110α kinase activity [24]. Homozygocity for this mutation resulted in embryonic lethality, while heterozygocity led to impaired signaling via the insulin-receptor substrate (IRS) proteins, which are key mediators of insulin, insulin-like growth factor I (IGF-I) and leptin action. As a result, the mutant mice displayed reduced somatic growth, hyper-insulinaemia, glucose intolerance, hyperphagia, and increased adiposity. Another study recently defined the p110α subunit as the critical lipid kinase required for insulin signaling in adipocytes and myotubes [25]. The discovery of the role of p110α as a key intermediate in metabolic signaling raises concerns about potential side-effects of PI3K inhibitors.

The biological functions of the PI3K class I_A catalytic subunit p110β have been studied by a partial deletion allele knock-out mouse [26]. Targeting of this specific isoform resulted in very early embryonic lethality in the homozygous state. Zygotes with the $PIK3CB^{del/del}$ genotype were non-viable very early after fertilization, leading to a deficiency at the blastocyst stage [26]. Crossbreeding studies of p110β knock-out mice with p110α-deleted mice did not reveal any possible redundant functions of these two class I_A catalytic isoforms. However, a possible overlap in functions that might be manifested in more subtle phenotypical abnormalities could not be ruled out so far [26].

Mice deficient in the class I_A PI3K catalytic subunit p110δ have been described by three different groups [27-29]. These studies revealed a major function of p110δ in B and T lymphocytes and a failure of the knock-out mice to mount normal immune responses. The mice lacking p110δ showed a reduction in the amount of B1 and marginal zone B cells, as well as lowered serum levels of immunoglobulins [27]. Furthermore, antigen receptor signaling in B and T cells was impaired and the mice were prone to the development of inflammatory bowel disease [28]. However, despite the high levels of p110δ expression in hematopoietic tissues, no significant differences were found in either the number and morphology of erythrocytes, peripheral leukocytes and lymphocytes, or hemoglobin levels between wild-type and p110δ-deficient mice [29].

Knock-out mice with a targeted deletion of the PI3K class I_B catalytic subunit p110γ have been characterized by three different groups [11,30,31]. These studies described phenotypes mainly in components of the innate immune response. Mice lacking p110γ showed an accumulation of defective neutrophils, which exhibited a failure in their migratory capacity and a reduced thymic cellularity. Moreover, chemoattractant-stimulated signal transduction was inhibited, because of an impairment of PIP_3 production in p110γ-deficient cells [30]. In contrast to p110δ, the deletion of p110γ resulted in no effect on B cells. Instead, this isoform appeared to regulate proliferation and cytokine production of T lymphocytes [30]. In view of the impaired migration capacity of p110γ knock-out macrophages towards a wide range of chemotactic stimuli, a crucial role for macrophage accumulation in the inflammatory response was furthermore suggested [11]. Phenotypical analysis of mice expressing a kinase-dead p110γ only partially reproduced the phenotype of knock-out animals [32]. Whereas both mice exhibited an identical impairment in innate immune reactions, the p110γ-deficient mice additionally showed a basal enhancement of cardiac contractility and cardiac tissue damage upon pressure overload. It was therefore suggested that p110γ possesses a kinase-independent function in the control of cardiac responses. A role of p110γ in the control of heart function was also described by Crackower *et al.* [33]. These studies described a role for p110γ as a negative regulator of cardiac contractility through the inhibition of cAMP production.

Regarding the regulatory PI3K subunits, gene-targeting strategies have been aimed at disrupting the adapter subunits p85α, its splice variants p55α and p50α, as well as p85β. Ablation of the whole *PIK3R1* gene, which encodes p85α and the mentioned splice variants, resulted in perinatal lethality [34]. However, disruption of the *p85α* gene only led to impaired B cell development, a reduction in the number of mature B cells, reduced B cell proliferative responses and a lack of T cell-independent antibody production [35]. Homologous deletion of the gene encoding the adapter subunit p85β resulted in growth reduction of the knock-out mice, compared to their wild-type littermates [36]. Surprisingly, muscle and adipose tissues of both the p85α and p85β knock-out mice exhibited increased insulin-stimulated PI3K activation, enhanced translocation of the glucose transporter isoform-4 to the plasma membrane, as well as hypoglycemia with decreased plasma insulin [34, 36]. Moreover, the mice showed enlarged muscle fibers, brown fat necrosis and calcification of cardiac tissue [34].

PHOSPHOINOSITIDE 3-KINASES AND HUMAN DISEASE

As the PI3Ks are known to be involved in a wide spectrum of control mechanism in the cell, deregulation of their function has been linked to various human diseases. Key roles of the PI3Ks have so far been described in tumor formation and metastasis, chronic inflammation, allergy and cardiovascular disease.

The importance of PI3K signalling in human cancer is highlighted by the fact that there are numerous oncogenes and tumor suppressor genes whose deregulation activates PI3K signaling and enhances the malignant properties of cells [37]. A prominent finding in this context were mutations in the tumor suppressor gene phosphatase and tensin homologue (*PTEN)*, which have been described in various human tumors [1, 37-39]. PTEN is a phosphatase that antagonizes the action of PI3K by de-phosphorylating the D-3 position of polyphosphoinositides [40]. The DNA sequence copy number of *PIK3CA*, the gene encoding the p110α catalytic subunit of PI3K located on chromosome 3q26, is frequently increased in ovarian cancers [41]. Moreover, recent reports have described activating mutations in the *PIK3CA* gene in a variety of other human cancers, including, medulloblastoma, breast and colon cancer [42, 43]. Although mutational alterations have predominantly been characterized for p110α so far, an increase in p110β activity was also found in human colon cancer biopsies and adenocarcinoma cell lines [44]. Furthermore, a knock-down analysis of p110β in a prostate cancer mouse model suggested a role for this PI3K isoform in the formation of metastases [45].

Gene-targeting strategies have furthermore uncovered a role for p110γ in colorectal adenocarcinoma [46]. Lack of p110γ significantly increased the incidence rate of spontaneous development of multifocal carcinomas and invasive adenocarcinoma in the colon of mice. In humans, protein expression analysis of p110γ revealed a loss of this protein in a high number of colon cancer cell lines, as well as in primary adenocarcinomas isolated from the colon of human patients [46].

As shown by different studies [30, 47], p110γ also plays a pivotal role in mediating leukocyte chemotaxis and activation, as well as mast cell degranulation, thus suggesting an involvement in inflammatory diseases. A specific contribution of the p110γ PI3K isoform to inflammation was supported by a recent study demonstrating that p110γ-deficient mice were protected from rheumatoid arthritis [48]. These mice were essentially protected against collagen II-specific antibody-induced arthritis, correlating with the defective neutrophil chemotaxis observed in this knock-out model [48]. Moreover, treatment of different rheumatoid arthritis mouse models with orally active small-molecule inhibitors of p110γ suppressed the progression of joint inflammation and damage in these mice [48]. Other studies, however, have described a positive role for the PI3Ks in the inflammatory response. In rheumatoid arthritis, the anti-inflammatory cytokine IL-10 is spontaneously produced by macrophages and infiltrating blood lymphocytes in the rheumatic joint [49]. An involvement of PI3K signaling in the regulation of IL-10 is known [50] and further analysis demonstrated that inhibition of PI3K signaling suppressed the production of this cytokine [51].

The PI3K isoform p110δ has also been shown to be involved in the inflammatory response [27]. Recent studies further supported this finding and described a contribution to allergen-IgE-induced mast cell activation and vascular permeability, which are common characteristics of chronic inflammations, such as asthma [52]. Pharmacological inhibition of p110δ in a murine asthma model resulted in a significant reduction in IgE serum levels and in an attenuation of airway inflammation and hyper-responsiveness, by preventing vascular leakage [52, 53].

Gene knock-out studies of the class I_A regulatory subunits in skeletal muscle of mice revealed a significant reduction in muscle weight and fiber size. Moreover, these mice exhibited insulin resistance in the muscle and whole-body glucose intolerance [54]. The p85 regulatory subunits are therefore thought to act as critical mediators of PI3K signaling in the regulation of muscle growth and metabolism. Furthermore, they are thought to make an important contribution to the symptoms of hyperlipidemia associated with human type 2 diabetes [54]. Diabetes is associated with vascular complications, including the impairment of vascular function and alterations in the reactivity of blood vessels to vasoactive agents [55]. It has been shown that PI3K signaling plays a role in vascular growth, proliferation and apoptosis and is implicated in modulating vascular smooth muscle cell contractility [56]. A recent study demonstrated that selective inhibition of PI3K attenuated the development of diabetes-induced abnormal vascular reactivity in the carotid arteries of diabetic rats [57].

The growing understanding of the biological functions of PI3Ks has provided new insights into the links between signaling events and cellular responses. As a result, these kinases have become interesting targets in clinical research, driving the urge to patent. Patents were deposited on various components involved in, or targeting PI3K signaling, such as polynucleotides encoding PI3Ks [58], constitutively active PI3Ks and uses thereof [59], cDNA sequences of different PI3K isoforms [60, 61], signaling pathway transducers [62], pharmacological inhibitors [63], and various derivatives [64-68].

PATENTS

Cloning, Expression and Characterization of a Novel Form of Phosphatidylinositol-3-Kinase [60, 69]

Stoyanov *et al*. reported for the first time the cloning and characterization of a G protein- activated human phosphatidylinositol 3-kinase [70]. Here we will discuss the identification of the novel isoform of the PI3K family, termed p110γ and the cloning, expression and characterization of this enzyme. These findings were patented by the United States Patent Office and by the European Patent Office [60, 69]. The invention concerns a nucleic acid sequence, encoding p110γ, an antibody directed against the protein, and discusses the diagnostic and therapeutic use of the protein, the nucleic acid sequence and the antibody. The invention comprises seven nucleic acid/protein sequences

(SEQ ID NO: 1-7) and eleven claims regarding the use of these sequences.

The major subject matter of the invention is a nucleic acid sequence encoding a novel PI3K isoform, p110γ. In order to isolate the cDNAs, a human bone marrow cDNA library was screened using the polymerase chain reaction. A human bone marrow cDNA library was chosen based on the findings by two other groups, which identified a novel PI3K activity in purified neutrophils and platelets stimulated by G protein βγ subunits [71,72]. For the library screening, oligonucleotide primers were generated based on the highly conserved amino acid sequences KNGDDLR (termed SEQ ID NO.6) and HIDFG (termed SEQ ID NO.7), which were reported for the catalytic subunit of the first cloned and characterized PI3K (residues 803-809 and 932-936 on p110α) [73]. Using these primers, a 402 bp-long fragment was obtained and subsequently subcloned and sequenced. The PCR product showed a 57% homology to the corresponding region of the gene encoding the bovine p110α. These PCR fragments were further used as a probe for the identification of a set of overlapping clones from a human U937 cDNA library. These overlapping clones were isolated and analysed, which led to the identification of the largest clone, containing the nucleic acid sequence termed by the authors SEQ ID NO.1. This cDNA sequence contained an open reading frame which codes for a protein of 1049 amino acids (termed SEQ ID NO.2). The new protein sequence was termed p110γ and displayed a molecular weight of approximately 120 kDa, similar to the previously published catalytic isoforms p110α and p110β [73,74]. Furthermore, two other highly similar nucleic (SEQ ID NO.3) and amino acid (SEQ ID NO.4) sequences were obtained from the cDNA library, which code for p110γ with 1050 amino acids. Thus the invention encompasses two nucleic (SEQ ID NO.1,3) and two amino acid (SEQ ID NO. 2,4) sequences coding for human p110γ. It further includes naturally occurring allelic human variations of p110γ, as well as proteins produced by recombinant DNA technology, which correspond to the proteins encoded by these sequences.

Furthermore, sequence homology analysis revealed that the amino acid sequence of p110γ has a homology of 36% to the human p110α, 33.5% to the human p110β and 27.7% to the yeast PI3K Vps34. The 400 C-terminal amino acids comprised a highly conserved region in these PI3Ks, which encodes a putative catalytic domain. The authors reported moreover that there was no significant homology in the amino-terminal regions of these PI3Ks, which in p110α is known to be responsible for the binding to the regulatory subunit p85α and p85β [75]. This finding raised the question of a different regulatory mechanism for p110γ.

A further subject of the invention is the detection of p110γ at the protein and transcriptional level. The availability of an antibody that recognizes epitopes in specific regions of p110γ would be a big advantage for the detection of the protein in biological samples and may be applied in diagnostic techniques, where patient samples could be tested for abnormal levels of this protein. In order to detect the expression of p110γ at the protein level, a polyclonal rabbit antiserum against p110γ was produced by immunization with a 15 amino acid-long peptide corresponding to a unique sequence of p110γ NSQL PESFRVPYDPG (SEQ ID NO.5). The expression of p110γ in mammalian tissues was analyzed by immunoprecipitation with this specific antibody and subsequent Western blot analysis using the same antibody. In the human leukemic cell lines U937 and K562, p110γ was detected as a 110 kDa protein. Hence, a further subject matter of the invention is an antibody specific for p110γ, which does not show any cross-reaction with other PI3Ks.

Furthermore, the invention encompasses the detection of p110γ at the transcriptional level under stringent hybridization conditions. Multi-tissue Northern blot analysis was performed with random prime-labeled PCR fragments encompassing the sequences encoding amino acids 1 to 233 of p110γ. This analysis revealed different expression levels of a 5.3 kb-long mRNA in human tissue from pancreas, kidney, skeletal muscle, liver, lung, placenta, brain and heart.

The invention further encompasses the recombinant expression of p110γ. The cloning of the nucleic acid sequence coding for p110γ in principle allows the construction of an expression vector enabling the production of the p110γ protein in a suitable host cell. The authors presented the use of a baculovirus expression system for the expression of p110γ. The cDNA encoding p110γ was cloned from codon 4 into the pAcG2T baculovirus transfer vector (BD Biosciences), which allows foreign genes to be expressed as glutathione S-transferase (GST) fusion proteins. Sf9 cells were co-transfected with pAcG2T-p110γ and linearized baculovirus DNA prior to expression and purification of the recombinant protein according to standard protocols [75]. This method further allowed the characterization of the substrate specificity of p110γ, which was found to phosphorylate PI, PI(4)P and PI(4,5)P_2 on the D-3 position of the inositol ring and was inhibited by wortmannin at nanomolar concentrations. However, recombinant p110γ failed to bind the p85α or p85β regulatory subunits. Furthermore, the catalytic activity of the recombinant p110γ could be stimulated when incubated with the Gβγ subunits, or by Ras-GTP.

Based on the findings described above, the authors further presented a pharmacological model based on the p110γ protein, the antibody targeting it and the nucleic acid sequence coding for the active protein. These tools could be used for analyzing the expression and modulating the activity of p110γ, which could lead to alterations in cell proliferation, histamine release, differentiation, or glucose transport.

We will now present further insight into the importance of this patent, the increasing evidence of the biological importance of p110γ in human diseases and their treatment with novel specific inhibitors. After the purification of the catalytic p110γ and the regulatory p101 subunit from pig neutrophils [76], the crystallographic structure of the highly conserved catalytic subunit of the porcine p110γ was reported and revealed in detail the structure of the catalytic domain and the Ras-binding domain (RBD) of p110γ [77]. Since pharmacological inhibition of the PI3K activity by

wortmannin [16], or LY 294002 [18] was useful to demonstrate their role in a variety of leukocyte responses, but failed to discriminate between distinct PI3K isoforms, neutralizing, isoform-specific antibodies were used for this purpose. For the first time, such an inhibitory antibody was used to determine the role of p110γ in leukocyte function, which indicated that this enzyme is required for natural killer cell migration upon chemokine stimulation [78]. The fact that p110γ is the sole PI3K isoform activated by GPCRs *in vivo* has been definitively shown in p110γ knockout-mice. Three groups reported the generation of p110γ-deficient mice, which revealed that inactivation of p110γ allows normal embryonic and adult development, but causes defects in the immune system [11,30,31]. p110γ-deficient neutrophils showed defects in migration and oxidative burst in response to GPCR agonists. Furthermore, it was reported that p110γ controls thymocyte survival and activation of mature T cells, but has no role in the development or the function of B cells. Moreover, it was observed that p110γ-deficient macrophages have significantly reduced migration capacity towards chemotactic agents [11]. Taken together, these data demonstrate that p110γ has a crucial function in linking GPCR signaling to $PI(3,4,5)P_3$ production and hence controls the motility of neutrophils and macrophages. Since abnormal macrophage infiltration causes chronic inflammatory diseases, Hirsch *et al*. further proposed, that p110γ could be a suitable target for the development of novel specific inhibitors to modulate the function of inflammatory cells [11]. A subsequent study revealed a novel role for p110γ in the modulation of mast cell activation and the control of allergic and inflammatory responses [47]. Despite the crucial role of mast cells in the response to infectious agents and parasites, abnormal mast cell activation can cause allergy, asthma and ultimately anaphylactic shock. The release of histamine-containing granules and the increase in intracellular calcium concentration were both attenuated by the loss of p110γ. Furthermore, the existence of an autocrine activation loop leading to enhanced mast cell activation was proposed [47]. These observations pointed out that inhibition of p110γ could result in a therapeutic advantage by diminishing the development of chronic inflammatory diseases and the migration of inflammatory cells, since multiple chemokine agonists activate GPCRs which signal through p110γ. Based on the emerging evidence that leukocyte motility is crucial in inflammatory disease and that signal transduction occurs through p110γ, Fuchikami *et al*. presented the first high-throughput *in vitro* screen for p110γ activity. The assay made use of recombinantly expressed His-tagged human p110γ and had the potential for high-throughput screening for inhibitors of lipid kinase activity [79]. Such an assay could be very useful in identifying novel specific p110γ inhibitors, due to fact that the catalytic activity of p110γ is crucial for leukocyte migration. It has indeed been reported that mice expressing catalytically inactive p110γ show the same chemotactic defects as those observed in $p110\gamma^{-/-}$ mice [32]. The application of novel specific p110γ inhibitors identified by improved screening approaches resulted in the successful treatment of two chronic inflammatory diseases: systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) in mice [48, 80]. These results revealed for the first time the high efficacy of targeting signaling molecules in chronic inflammatory disorders and raise hope for developing therapies with diminished side effects and increased life quality for patients with chronic inflammatory diseases. The use of these specific p110γ inhibitors further revealed the contribution of this isoform to thrombus stability [81]. Hence the development of anti-thrombus therapies based on recent findings that p110γ modulates the formation and stability of multi-platelet aggregates will be of interest [81-83].

Surprisingly, recent studies have also revealed an oncogenic potential for wild-type p110γ. It was shown that over-expression of this isoform induced oncogenic transformation in chicken embryo fibroblasts and moreover, that the lipid kinase activity of p110γ was essential for transformation [84]. However, cancer-specific mutations in p110γ have not been reported to date [84].

More recently, a study revealed a crucial role of p110γ in electrotaxis-regulated wound healing. Targeting the p110γ gene in mice led to a decrease in electrical field-induced signaling and attenuated the directed migration of healing epithelium [85]. Thus, the first gene involved in cell movement and wound healing in response to electrical signals was identified, making p110γ a very promising tool to modulate the wound healing process.

Ten years after the discovery of p110γ, studies on this key enzyme still uncover new biological functions and the initial hypothesis that targeting this enzyme could have a significant therapeutic benefit could be confirmed by studies using novel specific pharmacological inhibitors.

Phosphatidylinositol 3-Kinase p110δ Catalytic Subunit [61, 86] and Inhibitors of Phosphatidylinositol 3-Kinase p110δ [64, 87]

The class I_A PI3K catalytic subunit p110δ was identified and isolated as the last isoform within this class of PI3Ks [6]. A patent on polynucleotides encoding p110δ and recombinant p110δ polynucleotides, antibodies to p110δ and assays for identifying inhibitors of p110δ was issued in 1999 by Chantry *et al*. [61, 86]. Their invention was initially based on the identification and isolation of a novel lipid kinase closely related to the other class I PI3K isoforms. The enzyme displayed substantial sequence homology with p110β (72%), p110α (49%) and p110γ (45%) in the carboxy-terminal catalytic domain. The newly identified cDNA sequence encoding the lipid kinase was designated p110δ and classified in the class I_A of the PI3K family.

The cloning and characterization of p110δ was based on a strategy of amplification of conserved PI3K cDNA sequences. In a PCR analysis, degenerate oligonucleotide primers based on conserved sequences in the catalytic domain of known PI3Ks were used to screen a human cDNA library [6]. The identified full length human p110δ cDNA comprised an open reading frame of 3135 nucleotides, encoding a protein of approximately 114 kDa. Similarly to p110β, the catalytic domain of p110δ was found to be located in the C-terminus of the protein. The functionality of the newly identified PI3K was shown by a lipid kinase activity assay after transfection of cells with epitope-tagged

p110δ. Production of PI 3-phosphates demonstrated a functional PI3K enzyme activity, which was sensitive to the PI3K inhibitor wortmannin. Similarly to the other class I_A PI3Ks, which are known to form heterodimers with a p85 subunit, co-immunoprecipitation studies revealed the ability of p110δ to associate with the adapter protein p85. Expression analysis in various human tissues revealed an abundant distribution of p110δ in lymphocytes and lymphoid tissue, suggesting a role in the immune system [6].

The identification of p110δ as another member of the PI3K family opened an interesting avenue for further investigations. The whole family of PI3Ks has been shown to be of high importance in the control of various cellular responses, which has made them interesting targets for research and clinical applications. The described patent includes various of these possible applications.

As a protein involved in signal transduction, the p110δ polypeptide is an interesting target for interaction studies. Its use involves the generation of fragments modulating the binding of p110δ to a binding partner, or analogs with additions, substitutions or deletions in order to increase or decrease the binding affinity of p110δ to a binding partner. Furthermore, modification of the polypeptide might facilitate its passage into the cell and help control its localization. Antibodies specifically immunoreactive with p110δ provide another useful tool to modulate the binding affinity of p110δ and can further be used for purification of the protein, its detection and quantification in biological samples. Another aspect is that the understanding of the polypeptide provides a base for the identification of modulators that affect the binding affinity, or the functionality of p110δ, as well as the expression levels of the protein.

With respect to human diseases, the chromosomal localization of certain genes is of high interest to identify deregulated expression of their transcripts. As elevated function of PI3Ks has been shown to play a role in carcinogenesis, the polynucleotides described in the invention may be useful tools for chromosomal localization studies. Furthermore, the above described antibodies could constitute valuable diagnostic tools.

A patent on methods for inhibiting p110δ activity, and methods for treating diseases was deposited by Sadhu *et al.* in 2003 [64, 87]. The methods employ active agents that selectively inhibit p110δ, while not significantly inhibiting other PI3K isoforms. The applications of these p110δ inhibitory compounds are of high interest for the treatment of diseases such as disorders of immunity and inflammation. These inhibitors could also be used to inhibit cancer cell growth and proliferation. Furthermore, methods for the inhibition of p110δ-mediated processes *in vitro* and *in vivo* were included in the described patent.

Generic PI3K inhibitors such as LY294002 [18] or wortmannin [16, 17] have contributed a lot to the understanding of these kinases. The identification of specific functions of the different PI3K isoforms, however, requires more targeted approaches and forced the quest for substances acting on only one specific enzyme. The described invention provides the structure of several compounds displaying selective inhibitory activity towards p110δ. The compounds were selected to be at least 10-fold more selective for p110δ, when compared to other PI3K isoforms. The described compounds inhibit the biological activity of human p110δ and represent a useful tool to further characterize the functions of p110δ. Another aspect of the invention is that the compounds provide methods to selectively modulate p110δ activity, which represent useful tools for the treatment of diseases caused by p110δ dysfunction.

An embodiment for the use of the described patent includes methods for inhibiting the growth and proliferation of cancer cells. The PI3K p110δ has been suggested to play a role in cancer development and a study has shown that p110δ enhances the radiation-induced tumor control in murine cancer models [88]. Treatment of mice with Lewis lung carcinoma or hind limb tumors with a p110δ-specific inhibitor in combination with radiation abrogated the radiation-induced phosphorylation of Akt and significantly reduced the tumor volume [88]. A recent study further underlined the potential of p110δ-specific inhibitors in cancer treatment by describing a reduction in cell proliferation of acute myeloid leukemia (AML) cells [89]. Treatment of AML cells with a p110δ-specific inhibitor decreased the constitutive phosphorylation of Akt found in AML cells and furthermore was synergistic in reducing cell viability in combination with the topoisomerase II inhibitor VP16 [89]. Another field of application results from the known role of p110δ in B cells. Studies have shown an involvement of p110δ in the recruitment and activation of certain inflammatory cells [90]. Inhibition of p110δ impaired the movement of neutrophils across inflamed venules [90]. Furthermore, specific inhibition of p110δ activity attenuated the allergic airway inflammation and hyper-responsiveness in a murine asthma model [52]. Treatment of model mice with a p110δ-specific inhibitor significantly reduced antigen-induced airway infiltration of inflammatory cells, secretion of T_H2 cytokines in the lungs, as well as vascular permeability [53].

Mutations of the *PIK3CA* Gene in Human Cancers [91], Condensed Heteroaryl Derivatives and their Use as Inhibitors [65]

The p110α isoform was described as a retroviral oncoprotein that can transform chicken embryo fibroblasts *in vivo* [92]. Its key role in tumorigenesis was further supported by accumulating evidence indicating that p110α gain-of-function by over-expression or by somatic missense mutations is common in many human cancers. The *PIK3CA* gene consists of 20 exons encoding a protein of 1068 amino acids and 124 kDa size. The 3q26 locus where it is located is amplified in several human cancers, including head and neck cancers [93], cervical cancers [94], gastric cancers [95] and lung cancers [96]. In a landmark study, Samuels *et al.* performed a large-scale sequence analysis of 8 PI3K and 8 PI3K-related genes in human cancers and discovered that *PIK3CA* was the only gene harboring somatic mutations [43]. Furthermore, they reported a surprisingly high proportion of colorectal cancers (32%), gastric cancers (25%) and glioblastomas (27%) carrying somatic mutations in *PIK3CA*. These mutations also occurred in a smaller fraction of lung cancers (4%) and breast cancers (8%). Interestingly,

all mutations were apparent activating missense mutations that were found to cluster primarily in two major "hot-spots", E545 in the helical phosphatidylinositol kinase homology domain, and H1047 in the catalytic domain (exon 9 and exon 20, respectively). Clearly, the clustering of mutations implies that *PIK3CA* sequence analysis may prove useful for diagnostic purposes, as a marker for early detection of cancers, or for monitoring tumor progression. Furthermore, these data provide a reasonable case for considering p110α as a potential target for pharmacological intervention. Subsequent studies expanded these findings and collectively found *PIK3CA* mutations in a wide range of tumors, such as modulloblastoma (5% and 27%), medulloblas-toma (5%), breast cancer (18%, 25%, 27% and 40%), lung cancer (4% and 1%), gastric cancer (6.5%), ovarian cancer (6.6% and 12%), liver cancer (36%) and acute leukemia (1%) [43, 97-100].

Samuels *et al*. patented their discovery [91], which comprises methods for assessing cancer on body samples from a human suspected of having cancer based on the detection of intragenic mutation in a *PIK3CA*-coding sequence. The invention also includes several methods for inhibiting progression of a tumor in a human by reducing the expression, or inhibiting the activity of p110α, a method for delivering an appropriate chemotherapeutic drug to a patient in need thereof, and a set of one or more primers for amplifying and/or sequencing *PIK3CA*.

The discovery of non-random somatic mutations in the *PIK3CA* gene in a wide range of human tumors strongly pointed to an oncogenic role for the mutated enzyme. In order to elucidate the consequences of *PIK3CA* alterations, Kang *et al*. determined the growth-regulatory and signaling properties of the three most frequently observed PI3K mutations: E542K, E545K, and H1047R. When expressed in chicken embryo fibroblasts, all three mutants induced oncogenic transformation with a high efficiency and led to constitutive increases in the phosphorylation of Akt, p70 S6 kinase, and 4E-binding protein-1 [42]. This transforming ability correlated with elevated catalytic activity in *in vitro* kinase assays. The expression of these mutant *PIK3CA* enzymes conferred Akt activation in the absence of growth factor stimulation, promoted cell growth and invasion [42, 101]. Moreover, treatment with the PI3K inhibitor LY294002 abrogated PI3K signaling and preferentially inhibited growth of *PIK3CA* mutant cells.

This remarkable progress in our understanding of the role of the PI3K p110α in tumorigenesis allowed the development of new therapeutic strategies for the treatment of cancers harboring *PIK3CA* gene amplifications or mutations. Recently Hayakawa *et al*. carried out a high-throughput screen to identify novel p110α inhibitors and 4-Morpholino-2-phenylquinazolin was discovered as having p110α - inhibitory activity [102]. A series of derivatives were synthesized from this lead compound. Introduction of a 3-hydroxy group on the phenyl group and replacement of the quinazoline ring with a thieno [3,2-*d*]pyrimidine ring resulted in 3-(4-Morpholinothieno[3,2-*d*]pyrimidil-2-yl) phenol hydrochloride, also designated YM024. This compound is a highly selective inhibitor of p110α, when compared to other kinases and PI3K isoforms, with an IC_{50} value of 2.0 nM, making it the first example of a p110α-selective PI3K inhibitor. These series of compounds were patented in 2003 by the same group [65], claiming a pharmaceutical composition for a PI3K inhibitor comprising a fused heteroaryl derivative, or a salt thereof and a pharmaceutical acceptable carrier. Moreover, patents exist for their use in the manufacturing of medicaments for use in the treatment of a disorder in which PI3K plays a role, and for use in the treatment of cancer. They also describe a method to treat disorders which are associated with PI3K and to treat cancer by administering an effective amount of the fused heteroaryl derivative to a patient [65]. According to the invention, the pharmacological effects of the compounds have been verified by several pharmacological tests. Compounds of the present invention exhibited an excellent p110α-inhibitory activity, with IC_{50} values below 1 μM, whereas others were confirmed to have inhibitory activities against other PI3K isoforms, such as PI3KC2β. Several compounds exhibited an excellent growth-inhibitory activity *in vitro* against colon cancer, melanoma, lung cancer, glioma, ovary cancer, prostate cancer and pancreas cancer. Furthermore, inhibition of cancer cell growth was assessed *in vivo*. HelaS3 cells, a human cervix cancer cell line, were inoculated into the flank of female BAlb/c nude mice by subcutaneous injection. When the tumor reached 100-200 mm^3 in volume compounds were administrated intraperitoneally once a day for 2 weeks and tumor volume after treatment was determined. The test compounds exhibited superior anti-tumor activities as compared the control group.

YM024 has also already proven to be a valuable research tool for the investigation of isoform-specific p110α functions, not only in cancer, but also in other PI3K-mediated processes, such as inflammation [103].

PIK3CA represents one of the most frequently mutated oncogenes identified to date and one of the few established human oncogenes that is commonly activated through either gene amplification or point mutations. Notably, no mutations in the other PI3K catalytic subunits have been reported in human cancer until the present date, suggesting that the PI3K p110α harbors a selective oncogenic potential. The oncogenic transforming activity of the mutant p110α proteins makes them promising targets for small molecule inhibitors that could be further developed into effective and highly specific anticancer drugs.

Methods for Modulating T cell Responses by Manipulating Intracellular Signal Transduction [104]

T lymphocytes are cells involved in the adaptive immune response (secondary response), which in case of infection are able to provide a more specific response than the innate immune system (primary response). T cell precursors produced by the bone marrow, migrate to the thymus, where they undergo complete differentiation and selection. In the thymus, T cells come in contact with self MHC-peptide complexes expressed on antigen-presenting cells (APCs) to which the T cells should not react. T cells that show the appropriate affinity for the self MHC-peptide complexes are allowed to migrate to the secondary lymphoid organs (spleen and lymph nodes), while the others are eliminated [105]. Immature T lymphocytes express both the CD4 and CD8 co-

receptor molecules on their surface, but after the selection and maturation process they express only one of them. While $CD4^+$ cells recognize non-self antigen presented on the surface of APCs by MHC class II molecules and become cytokine-secreting cells, $CD8^+$ cells recognize non-self antigen presented by MHC class I and become cytotoxic effector cells. APCs include cells such as dendritic cells, Langerhans cells and natural killer cells (NK).

In the secondary lymphoid organs, the lymphocytes are in a state of anergy, until they encounter APC cells, which present a non-self peptide in complex with MHC class I or II molecules on their cell surface (either from a pathogen or a cancer cell) [106]. Mature T cells recognize MHC-non self peptide complexes via their transmembrane T cell receptor (TCR). However, this interaction is not sufficient to generate a proper immune response against the antigen. For complete T cell activation, an interaction with co-stimulatory ligands, such as CD80 and CD86 expressed on the surface of the APCs is necessary. These co-stimulatory ligands are able to bind to the CD28 receptor on the surface of T cells. The signals from this co-stimulatory receptor, in combination with those from the TCR, activate the cell and initiate gene transcription, cell growth and mitosis. One of the most important effects of the co-engagement of TCR and CD28 receptors is the production of IL-2, a cytokine that provides strong proliferative and survival signals.

Several studies have provided clear evidence for a rapid recruitment and substantial activation of PI3K in T cells, which drives the production of D-3 phosphoinositides as signal mediators. Activation of T cells induces a time-dependent elevation of the described second messengers, which remain high until nine hours after activation and then return to normal levels. Subcellular localization studies revealed a major presence of these phosphoinositides in the contact area between the T cell and the APC [107].

Further evidence for an involvement of PI3Ks in proliferation signals in T cells was provided by the fact that mouse T lymphocytes stimulated with an anti-CD3 antibody showed a decrease in their proliferation rate in the presence of the PI3K inhibitor LY294002. In T cells from mice carrying a mutation in the PI3K catalytic subunit p110δ ($p110\delta^{D910A/D910A}$), a reduction in Akt/PKB phosphorylation, as well as in $CD4^+$ T cell proliferation in response to CD3 stimulation was observed [28,108]. The proliferation capacity of $CD4^+$ cells was restored upon co-stimulation with CD28. These results supported other studies describing an important role for PI3K in TCR signalling independently of CD28, even though the molecular mechanism by which the TCR is coupled to PI3K is still unclear [109,110]. Similar experiments performed in p110δ knock-out mice yielded less clear results, which might be caused by a greater capacity of p110α and p110β to compensate for the absence of p110δ expression [29].

Interestingly, it was shown that CD28 can activate PI3K independent of the TCR and that CD28 contains a Tyr-X-X-Met motif in its cytoplasmatic domain that can bind the SH2 domain of p85 [107,111,112]. However, the exact role played by CD28 and its cytoplasmatic domain in the activation of PI3K is still not totally understood. CD28-deficient mice expressing a CD28-Tyr170Phe mutant (Phenylalanine to Tyrosine substitution in the PI3K binding motif of CD28), were still able to co-stimulate T cell proliferation and IL-2 production independently of CD28 association with PI3K [113]. Instead, a defect in Bcl-Xt activation and T cell survival was observed [113, 114]. On the other hand, the capacity of CD28-deficient mice to proliferate and produce IL-2 was also restored by expression of an activated mutant of Akt/PKB, which seems to be in conflict with the previous observation [115]. Despite the fact that the ability of CD28 to recruit PI3K might be dispensable for T cell proliferation and IL-2 production, it is still possible that CD28 can influence the capacity of the TCR to couple to PI3K and activate Akt/PKB independently of the CD28-Tyr170. Furthermore, the cytoplasmic domain of CD28 contains two Proline-rich regions (PYAP) that are able to facilitate the recruitment of p85 through its SH3 domain [116,117]. Taken together, it appears that the activation of PI3K due to CD28 triggering can be *direct*, through the involvement of the YXXM region (Fig. (**4**)), or *indirect*, through the involvement of the PYAP region and consequent involvement of proteins such as Lck and ZAP-70.

A patent on methods for modulating T cell responses by manipulating intracellular signals associated with T cell co-stimulation was deposited by June in 2004 [104]. The invention focuses on the inhibition or the activation of the production of D-3 poly-phosphoinositides in T cells, which can be achieved by manipulating PI3K signaling, or other intracellular signals associated with co-stimulation. The patent describes useful tools for clinical applications, including situations where it is desirable to inhibit an immune response to an antigen(s), e.g. organ or bone marrow transplantation and autoimmune diseases.

The evidence that CD28 activation plays a crucial role in T cell activation and that PI3K plays a central role in the CD28 cascade focused the attention of research on the possibility of suppressing the immune system by modulating PI3Ks and the signal cascade controlling its activation. The best described PI3K inhibitors are wortmannin [16,17] and the quercetin derivative LY294002 [18]. However, their lack of isoform specificity, as well as their high overall toxicity limits their utility with respect to clinical applications. In view of the limitations of generic pharmacological inhibitors, there is a need to find other agents that can be used alone, or in combination with these PI3K inhibitors. A main result of combining different drugs that act by different mechanisms is that a very powerful immunosuppressive effect can be achieved. This makes it possible to administer low doses of each single drug, which reduces drug-related toxicity. The use of compounds displaying higher specificities, such as inhibitors of the PI3K isoforms p110δ or p110γ would also have great advantages and are described in this review [60, 64]. An important benefit of specifically suppressing the immune system is that the body is still able to maintain a sufficient level of host defense to protect itself against infections and malignancies.

A new strategy directly connected to the suppression of CD28 activation was derived from the evidence that the cytotoxic T lymphocyte antigen 4 (CTLA4) receptor binds to

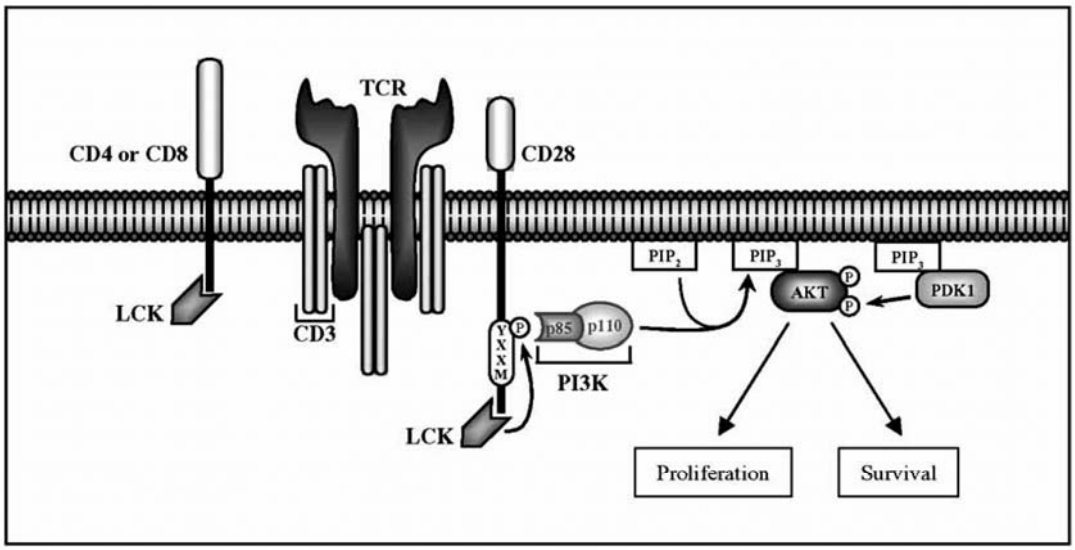

Fig. (4). Involvement of PI3K in T cell receptor (TCR) signaling. The cytoplasmatic tail of the CD28 co-receptor contains a YXXM motif, to which the PI3K regulatory subunit p85 can bind upon tyrosine phosphorylation. While PI3Ks have the potential to regulate different signaling pathways in T cells, the exact role they play is not yet clear.

the same APC ligand (CD80-CD86) as CD28 [118,119]. The interaction results in an inhibition of IL-2 production, cell-cycle progression and the activation of TCR-induced cyclins through blockage of JNK, Erk and NF-κB activation [120-122]. It would thus be possible to block CD28 engagement and induce CTLA4 triggering by treating patients with a B7-specific fusion protein CTLA4-Ig. The use and toxicity of this molecule is, however, a controversial issue and thus it was used in different approaches depending on the disease. In human bone marrow transplantation, CTLA4-Ig has an *in vitro* use to prevent graft-versus-host disease (GVHD). Its use *in vivo* is common in case of allograft rejection of kidney and heart, as well as for the treatment of psoriasis [123,124]. The effect of CTLA4-Ig might further be improved by combined use with a CD40L-specific monoclonal antibody that induces CTLA4 over-expression [125,126].

Several other drugs/agents are already under study, some of which directly target the different steps of the extracellular signal-regulated kinase (Erk) cascade, Jun N-terminal kinase (JNK), or p38 MAPK and are commonly used *in vitro* for interference with cytokine production. Current immune suppressive therapies which are used mainly in organ and bone marrow transplantation and occasionally in rheumatoid arthritis are Cyclosporine A (a fungal metabolite extracted from *Tolypocladium inflatum gams)* and Tacrolimus (derived from the soil fungus *Streptomyces tsukubaensis)*. These two molecules are able to block the translocation of the nuclear factor of activated T cells (NFAT) to the nucleus. In this way these compounds block IL-2 production and cell proliferation, which are the first steps in T cell activation [127-129].

All these different inhibitory approaches can be combined with the PI3K inhibitors described before. In this way, the problems resulting from the use of wortmannin or quercetin-derived inhibitors alone could potentially be overcome.

There are two main types of applications for inhibitors of T cell activation, which are *in vivo* and *in vitro*. In the first case, the PI3K inhibitor, together with the other agents chosen (especially in the case of antibodies), are directly injected into the patient. For example, in case of organ transplantation, the agents are injected together with the transplant and maintained by follow-up injections. However, this approach is not always successful, depending on the toxicity of the agents and possible unspecific collateral effects on other cells. In the case of transplantation, an *in vitro* approach is therefore often preferred. In this case, a state of anergy has to be induced in order to avoid the GVHD which happens in allogeneic transplantation. This approach is based on cells from a donor cultured together with a CD3-triggering agent and a CD28 inhibitor. At the time when the T cells are totally anergic, they can be transplanted into the patient together with the cells needed.

Most of what has been described above relates to the capacity to trigger CD28 and its related proteins in order to induce T cell anergy. However, these approaches can also be used for immune system stimulation, which is especially beneficial to induce tumor rejection. The basis of this approach is the possibility to produce a stronger co-stimulation for specific T cells that have already received a primary activation signal through TCR triggering. The most important application of PI3K activation/co-stimulation is in cancer immune therapy. In general, chemoresistant cancer cells that express specific antigens on their surface are able to induce T cell activation. However, this activation is often too slow and too low for a complete elimination of the cancer cells [130]. An approach to boost the signal for the immune system is the implementation of a co-stimulation,

independently of the presence of APCs. Some drugs already commonly used in the clinic are thalidomide analogs such as ImiDs and SelCIDs. Their role in co-stimulation of T cells has already been demonstrated [131]. As already discussed in the case of immune system tolerance, the use of these compounds can be planned *in vitro* or *in vivo*.

In vitro cancer immunotherapy can be divided into passive and adoptive methods, depending on the characteristics of the lymphocytes generated and injected into the patient. In the case of the adaptive immunotherapy, lymphocytes are cultured in the presence of a specific antigen and a CD28 stimulator (or agents that induce co-stimulation). In this case, the T lymphocytes generated and re-injected into the patient are able to directly attack the tumor cells [132, 133]. In contrast, lymphocytes generated for the active immunotherapy are not themselves specific for the antigen, but are able to stimulate the patient's immune system in order to respond better and faster to the antigen. The latter method can also be used in patients with a temporary immune deficiency in order to generate a temporary immune protection against common pathogens [134].

In vivo immunotherapy relates to the possibility of inducing a stronger and faster immunity without any cellular vaccination. Patients are treated with direct injections of agents which provide a stronger and longer co-stimulatory signal by acting directly on specific T cells that have already received a signal through *in vivo* TCR engagement.

The growing understanding of PI3Ks and their possible role(s) in the immune response opened an interesting field of research regarding the identification of better and safer immunosuppressive or immunostimulatory drugs. However, an improvement in short and long term outcome of the desired immune response is still needed. Multiple potential PI3K modulators have recently been described and screening assays will hopefully reveal their potential uses in immunotherapy. However, *ex vivo* studies still have to be performed for a better understanding of the capacity of drugs targeting PI3K activity to modulate immune responses. Systematic studies in animals and human patients will then be required to bridge the transition in drug development from target enzymes to the modulation of lymphocyte function.

CURRENT & FUTURE DEVELOPMENTS

The patents described in the present review have a wide range of potential applications in medical research, including the development of novel anti-cancer drugs and anti-inflammatory agents. This is the consequence of the various biological functions of distinct PI3K isoforms, which have been characterized in the past years. Gene targeting and transgenic mouse models have been instrumental in describing the distinct roles of the p110γ and p110δ isoforms in the immune system. These studies have been confirmed by the use of isoform-specific pharmacological inhibitors, which are now further being developed as novel drugs for diseases such as rheumatoid arthritis and allergies. In the case of p110α, the discovery of somatic mutations in the *PIK3CA* gene in a substantial number of different human cancers has made this PI3K isoform a validated target for the development of anti-cancer agents. However, in view of the complexity of the signaling networks involving PI3K isoforms [24,32,135], it remains unclear how soon drugs targeting these enzymes will successfully achieve the transition from the bench to the bedside.

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5.3 Targeting receptor tyrosine kinase signaling in acute myeloid leukemia

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Targeting receptor tyrosine kinase signaling in acute myeloid leukemia

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Contents

Abstract

Acute myeloid leukemia (AML) is a quickly progressing, heterogeneous clonal disorder of hematopoietic progenitor cells. Significant progress in understanding the pathogenesis of AML has been achieved in the past few years. Two major types of genetic events are thought to give rise to leukemic transformation: alterations in the activity of transcription factors controlling hematopoietic differentiation and activation of components of receptor tyrosine kinase (RTK) signaling pathways. This has led to the development of promising new therapeutic strategies for the disease. In this article, we will discuss recent developments in the field of molecularly targeted therapies for AML, which involve RTKs such as FMS-like tyrosine kinase 3 (Flt3), c-Kit and signal transduction via the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. Initial results imply that targeting RTKs is a very promising approach for AML and that other receptors, such as the insulin-like growth factor receptor (IGF-IR), could also represent new targets in the future.

Keywords: AML; Receptor tyrosine kinase; Flt3; c-Kit; IGF-IR; PI3K; mTOR

1. Introducing remarks

Acute leukemias represent a small (<3%) fraction of all human cancers, but are a leading cause of cancer-related

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mortality in children and young adults [1,2]. Acute myeloid leukemia (AML) accounts for about 25% of all leukemias in adults in the Western world, and is the second most frequent form of leukemia following chronic lymphocytic leukemia (CLL) [1]. The incidence of AML is 3.4 cases per 100,000 general population. AML is a heterogeneous group of diseases characterized by uncontrolled clonal proliferation of hematopoietic precursor cells leading to impairment of normal hematopoietic cell production, causing cytopenias. During the past decade, there has been a shift in AML diagnosis, classification, and treatment strategies from protocols relying on cell morphology and cytochemistry, to cell-surface antigen expression and the use of cytogenetic and molecular markers. Treatment of AML represents a difficult challenge, because of relapse and complications associated with treatment [2]. In the past 40 years, advances in supportive care and development of chemotherapeutic agents have led to improved outcomes in patients with acute myeloid leukemia. High relapse rates following remission have led to extensive efforts to develop techniques and regimens for detecting and eliminating minimal residual disease. Since standard cytotoxic chemotherapies have side effects to which AML patients, especially those of advanced age, are susceptible, there is a need to develop new therapies to overcome these difficulties [1,3]. A better understanding of the biology and the molecular pathogenesis of AML has led to the development of new, targeted agents and strategies for AML treatment. Targeted therapy has indeed improved outcomes in some AML patients. Most of the new agents used for AML therapy are less toxic than their predecessors, and combinations with more intensive traditional regimens are being tested.

2. Acute myeloid leukemia

2.1. Pathogenesis

Great efforts have been made to gain a better understanding of the molecular pathogenesis of AML. The malignancy is characterized by an accumulation of granulocyte or monocyte precursors in the blood. This is caused by the clonal growth of immature progenitor cells, which display increased proliferation and resistance to apoptosis, as well as impaired differentiation [4].

Recurring chromosomal translocations are a hallmark of human leukemias. There are more than 300 recurring chromosomal translocations, of which more than 100 have been cloned [5], providing important insights into the pathogenesis of disease. Chromosomal aberrations are found in about half of all AML cases and are grouped into two major subtypes: balanced and unbalanced aberrations [6]. The biology of AML with balanced chromosomal translocations is fairly well understood, in contrast to the situation in AML with unbalanced aberrations [6]. Moreover, nearly half of all AML cases present a non-aberrant karyotype [6,7]. In this category of AML, molecular analyses have revealed mutations in the *FLT3*, *CEBPA*, *KIT* and *NPM* genes, as well as *MLL* partial tandem duplication [6,7].

In AML, chromosomal translocations often result in loss-of-function mutations in transcription factors that are required for normal hematopoietic development [4,5,8]. The elucidation of the structure and function of leukemogenic genes, as well as the analysis of their occurrence in leukemia has led to a two-hit model of AML pathogenesis. According to this model, AML develops as a result of the concurrent emergence of two classes of genetic alterations (mutations or gene rearrangements). While class I mutations confer a proliferative and/or survival advantage to hematopoietic progenitors [4,5], class II mutations lead to impaired hematopoietic differentiation. The increased cell proliferation and survival of leukemic blasts (class I mutations) is thought to be caused by alterations in RTK signaling pathways. In this context, it has been demonstrated that mutations in FMS-like tyrosine kinase 3 (Flt3), c-Kit and Ras are frequent in AML [4,5]. The activation status of growth and survival pathways including phosphoinositide 3-kinase (PI3K)/Akt has also been found to be increased in AML blasts [9]. The impaired differentiation of AML blasts (class II mutations) is thought to occur through alterations in transcription factors required for normal myeloid cell differentiation [4,5,8]. In AML, these transcription factors are frequently the target of translocations, leading to the expression of fusion proteins such as AML1/ETO, CBF/SMMHC, PML-RARα, or MLL fusion proteins [4,5].

2.2. Genetic alterations involving transcription factors

Transcription factors are frequently targeted by balanced chromosomal translocations in AML. These include core binding factor (CBF), retinoic acid receptor alpha (RARα), and members of the HOX family of transcription factors [4,5,8]. Transcriptional coactivators, such as Creb-binding protein (CBP), p300, MOZ, TIF2, and MLL, are also targets of chromosomal translocations in AML [4,5]. The targeted transcription factors are thought to be important for normal hematopoietic development. The consequence of chromosomal translocations found in AML is loss-of-function and therefore impaired hematopoietic differentiation. For example, CBF is a heterodimeric transcription factor composed of a DNA-binding component, AML1, and the CBFß subunit which functions as a transcriptional activator of AML1. CBF controls the expression of genes that are critical for normal hematopoietic development. The three most common translocations involving CBF are the t[8;21], inv[16], and t[12;21] that result in expression of the AML1/ETO, CBFß/SMMHC, and TEL/AML1 fusion proteins, respectively. Each of these fusion proteins is a dominant negative inhibitor of CBF-mediated transcription. Transcriptional repression of CBF target genes by CBF-related fusion proteins is thought to be mediated through aberrant recruitment of the nuclear corepressor/histone deacetylase complex. Another example are fusion genes involving RARα, such as PML/RARα

associated with the t[15;17] translocation in acute promyelocytic leukemia. Expression of the PML/RARα fusion protein results in a block of differentiation at the promyelocyte stage caused in part by the recruitment of the nuclear corepressor complex. This block is relieved by all-*trans*-retinoic acid (ATRA), which induces the release of the nuclear corepressor complex by binding to PML/RARα. In addition to these direct inhibitory effects on transcriptional activation, the fusion proteins may also have additional roles in impairment of hematopoietic differentiation [5].

Interestingly, in hematological clonal disorders in children with Down syndrome (DS), mutations are commonly found in the gene encoding the transcription factor GATA-1 [10]. The incidence of childhood acute leukemia is highly increased in children with DS, with a prevalence of acute megakaryoblastic leukemia (FAB M7) [11]. GATA-1 is known to be critical for normal development of erythroid and megakaryocytic lineages, and the absence of GATA-1 promotes the accumulation of immature megakaryocytes [12]. The fact that mutations in GATA-1 occur in nearly every case of DS with a myeloproliferative disorder suggests that deregulation of GATA-1 is an essential step in the development of the disease in this particular patient group [13]. A direct link between trisomy 21 and the X-linked transcription factor GATA-1 has not been found so far. However, models including gene dosage effects have been proposed, and trisomy 21 is thought to cooperate with the non- or dysfunctional GATA-1 in the multi-step process in which progenitor cells acquire multiple genetic lesions [13].

3. Receptor tyrosine kinase signaling

Receptor tyrosine kinases (RTKs) are central components of cell signaling networks and play a crucial role in normal physiological processes such as in embryogenesis and development. RTK networks control fundamental cellular activities including cell proliferation and survival, cell cycle control, metabolism, as well as cell shape and movement. They are able to detect, filter, and process a variety of environmental and intercellular factors. Numerous membrane-spanning surface receptors have been identified and classified based on ligand preference, the induction of biological responses and their primary structure. Protein tyrosine kinases (PTKs) are endowed with intrinsic protein kinase activity that catalyzes the transfer of γ-phosphate of ATP to tyrosine residues of protein substrates [14]. The receptor tyrosine kinases (RTKs) are one large family of PTKs that contain an intracellular catalytic protein tyrosine kinase domain, an extracellular ligand-binding domain and regulatory sequences. More than 50 mammalian RTKs are known so far including the well known insulin receptor (IR), Fms-like tyrosine kinase 3 (FLT3), c-Kit, epidermal growth factor (EGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), colony-stimulating growth factor I (CSF-I) and insulin-like growth factor I (IGF-I) receptor [15]. A plethora of ligands such as peptides, proteins, lipids, or carbohydrates bind to and regulate the pleiotropic actions of RTKs. Tight control of the receptor activity is maintained by protein-tyrosine phosphatases, by other serine/threonine kinases, as well as autoregulatory mechanisms in the receptors. The extracellular binding of a specific ligand to a RTK induces conformational alterations that are translated across the membrane barrier resulting in the activation of its intracellular kinase activity [16]. In general, ligand binding induces allosteric interactions causing dimerization of receptor subunits through disulfide-bridges [17] and autophosphorylation of specific tyrosine residues within their cytoplasmatic domains [16,18]. Dimerization-mediated receptor activation mechanisms have been described for receptors composed of heterotetrameric structures such as the IR, IGF-IR, PDGFR and the EGFR. Tyrosine autophosphorylation stimulates the intrinsic kinase activity of the receptor and leads to the generation of recruitment sites for downstream signal transducers. Src homology-2 (SH2) [19] or phosphotyrosine-binding (PTB) [20] domains are known phosphotyrosine-recognition domains present in various signaling proteins.

Activation of RTKs leads to rapid stimulation of phosphatidylinositol metabolism and generation of multiple second messengers. One well characterized signaling pathway downstream of the IR and IGF-IR involves the insulin receptor substrates-1 to -4 (IRS-1 to -4) and the Src-homology collagen protein (Shc) isoforms as adapter molecules. The adapter proteins bind to the phosphotyrosine residues within the juxtamembrane region of the cytoplasmatic receptor and undergo subsequent tyrosine phosphorylation. Phosphorylation of the IRS adapter molecules on one hand triggers activation of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway [21–23], whereas, on the other hand, the Shc adapter activates signaling by the Ras/Raf/MAPK pathway (reviewed in [23]). Generally, signals transduced through those signaling cascades have pleiotropic effects on cell behavior controlling cell proliferation, differentiation and cell migration, as well as apoptosis [24]. The PI3Ks phosphorylate phosphatidylinositol (PI) on the D-3 position of the inositol ring, producing distinct second messengers such as PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 (PIP_3) [25]. These lipid products activate signal transduction by diverse proteins including phosphoinositide-dependent kinase-1 (PDK1), forkhead (FKHR), glycogen synthase kinase-3 (GSK-3), tuberous sclerosis complex 1 and 2 (TSC1, TSC2), BAD, Ras homologue enriched in the brain (Rheb), mammalian target of rapamycin (mTOR), 4E-binding protein (4E-BP) and ribosomal protein S6 kinase (S6K) [25]. The Ras/Raf/extracellular signal-regulated kinase (Erk) cascade proceeds from Ras and its downstream effector Raf to Erk (p42/44 MAPK) [26].

Protein tyrosine phosphorylation was discovered more than 25 years ago [27] and protein tyrosine kinases, the signaling pathways they activate, as well as the mechanisms underlying their action and regulation have been widely studied. The importance of understanding the complexity of

signaling networks, their regulation and the RTK interconnections has been highlighted by a large variety of molecular alterations and deregulations found in various human diseases including cancer.

3.1. Alteration of RTK signal transduction in AML

The increasing understanding of the pathogenesis of AML uncovered multiple genetic abnormalities necessary for the development of the disease. In the classical 'two hit' model, the second hit is thought to be caused by alterations in RTK signaling and various aberrantly regulated pathways have been described so far. Basically, there is an oncogenic potential in every receptor with tyrosine kinase activity. Structural modifications can lead to constitutive activation of RTKs, subversion of molecular control mechanisms and alterations in signal transduction. Deletions within the extracellular ligand-binding domain alter ligand responsiveness, or eliminate negative control mechanisms that this structure might exert on the kinase domain. Even point mutations are able to induce overall ligand-independent conformational alterations and hence activation of RTKs. Besides genetic alterations, over-expression of the wildtype receptor and/or autocrine receptor activation are known to play an important role in aberrant signal transduction.

The FMS-like tyrosine kinase 3 (FLT3) is the most frequently mutated gene in AML. About one-third of all patients show either internal tandem duplications (ITDs) within the juxtamembrane domain [28] of FLT3, or mutations within the activation loop, predominantly at position D835 [29]. FLT3 is a transmembrane receptor that has a crucial role in normal hematopoiesis. By signaling through the Ras/MAPK pathway the receptor exerts an important function in the control of cell proliferation. FLT3-ITD as well as activating loop mutations result in constitutive activation of the tyrosine kinase activity and of the whole signaling network [30,31].

Activating point mutations in the kinase domain of the human colony-stimulating factor (M-CSF or CSF-I) receptor FMS occur in 5–20% of patients with AML [32]. FMS is a cell surface RTK and specific point mutations in this receptor have been implicated in neoplastic transformation by inducing ligand independence and constitutive activation of the tyrosine kinase activity. The mutation at codon 969 of FMS is thought to alter a negative regulatory site of the receptor and to upregulate the response to ligand binding, conferring a growth advantage to the cell [32]. Co-expression of M-CSF together with FMS has been described for a small subset of AML patients, implicating autocrine stimulation of the wildtype receptor [33]. Moreover, patients with myeloplastic syndrome harboring FMS mutations were shown to have a significantly increased frequency of transformation to AML [34].

Mutations in the RTK c-Kit have been preferentially associated with AML exhibiting either an inv(16) or a t(8;21) karyotype [35]. c-Kit encodes a transmembrane receptor that is activated by stem cell factor (SCF). Studies have shown that the levels of activation of c-Kit correlate with the rate of proliferation of myeloid leukemia cells and that receptor over-activation contributes to the excessive proliferation and aberrant differentiation of AML cells [36].

Mutations in the *RAS* gene family have been described in about one-fourth of AML cases, predominantly in N-Ras, but also in K-Ras and, although very rarely, in H-Ras [37,38]. Primarily, mutations were found to affect the conversion of the active Ras-GTP form to the inactive Ras-GDP form leading to constitutively activated Ras and enhanced downstream signaling. Besides, over-activation of downstream transducers has been described as a result of FLT-ITD affecting the autonomous growth of AML cells [39]. Moreover, increased activation of the Ras/MAPK pathway has been shown to be caused by a commonly occurring chromosomal translocation. BCR-ABL is a chimeric oncoprotein that binds to Grb-2, the substrate protein known to link receptor tyrosine kinases to Ras signaling. Coupling of BCR-ABL-Grb-2 to SOS subsequently induces activation of the signaling pathway [40].

FLT-ITD has furthermore been associated with increased activation of the Janus protein tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. Constitutive tyrosine phosphorylation of the transcription factor STAT occurs in approximately 70% of AML cases, either through autophosphorylation of RTKs, or due to autocrine growth factor production [41]. STAT signaling involves the control of diverse biological processes including cell proliferation, differentiation, development, and survival. Abnormal STAT signaling has been implicated in oncogenesis [42]. Beside its role in FLT3 signaling, the JAK/STAT pathway has been associated with c-Kit-mediated ligand-independent survival and proliferation of AML cells. In fact, the c-Kit mutation Asp816 has been found to constitutively activate the JAK/STAT pathway [43]. Chromosomal translocations resulting in the fusion of the *JAK2* and the *TEL* gene have been described in human leukemias. The TEL–JAK2 fusion protein confers constitutive tyrosine kinase activity and cytokine-independent proliferation to hematopoietic cells [44,45].

An increasing body of evidence suggests a role of signaling by the IGF-IR through the PI3K/Akt/mTOR cascade in AML. Insulin-like growth factors (IGFs) together with other cytokines have been described to be important for AML cell growth [46] and autocrine IGF-I production has been suggested to influence drug resistance in an AML cell line [47]. Activation of the PI3K/Akt signaling pathway has been detected in blast cells from AML patients contributing to survival and proliferation of these cells [48,49].

4. Targeting receptor tyrosine kinase signaling in AML

Significant progress has been made over the past decades in the treatment of AML. Insights into the pathogenesis, detection of cytogenetic markers and classification of the disease have facilitated the definition of optimal therapy con-

Table 1
Pre-clinical studies in AML

Compound	Target	Cells/Xenograft models	Treatment schedule	Result	Refs.
Tyrosine kinase inhibitors					
Imatinib	BCR-ABL, c-Kit, PDGFR	MO7e, AML-4 12 primary patient blasts	0–10 μM for 90 min	Only marginal inhibition of cell growth Inhibition of SCF-induced c-Kit phosphorylation in MO7e	[78]
STI-571		HL-60/Bcr-Abl	0.25–0.5 μM for 48–72 h	Induction of apoptosis, inhibition of constitutively active Akt and NFκB, increased Ara-C or doxorubicin-induced apoptosis	[77]
Gleevec		FDC-P1 cells with Kit mutants cloned from AML patients	0–1 μM for 3–12 h	Dose-dependent growth inhibition and apoptosis in c-Kit mutant cells	[79]
SU5416	FLT3, c-Kit, VEGFR	MO7e 7 primary patient blasts	0–25 μM for 0.5–24 h	Inhibition of SCF-induced c-Kit and ERK phosphorylation in c-Kit$^+$ cells, inhibition of SCF-induced cell growth ($IC_{50} = 0.1$ μM) and induction of apoptosis	[54]
		BaF3, MV4-11, RS4;11	0–10 μM for 48–72 h	Inhibition of FLT3 autophosphorylation, inhibition of FLT3-dependent ERK and STAT5 phosphorylation, reduction in cell growth ($IC_{50} = 0.25$ μM) and induction of apoptosis	[55]
SU5614	FLT3, c-Kit, VEGFR, PDGFR	BaF3, MV4-11, RS4;11	0–10 μM for 48–72 h	Inhibition of FLT3 autophosphorylation, inhibition of FLT3-dependent ERK and STAT5 phosphorylation, reduction in cell growth ($IC_{50} = 0.1$ μM) and induction of apoptosis	[55]
SU11248	FLT3, c-Kit, VEGFR, PDGFR	MV4-11, RS4;11, OC1-AML5, Athymic nu/nu and nonobese diabetic-severe combined immunodeficiency (NOD-SCID) mice	0–10 μM 5–40 mg/kg/day	Inhibition of FLT3-driven phosphorylation and induction of apoptosis *in vitro*. Regression of FLT3-ITD tumors in subcutaneous xenograft mouse model and prolonged survival in bone marrow engraftment mode. Inhibition of FLT3-ITD phosphorylation for up to 16 h following single efficacious drug dose	[62]
PKC412	FLT3, c-Kit, VEGFR, PDGFR	BaF3 cells with FLT3 wt or mutant (-ITD, -D835Y) BaF3/FLT-3-ITD Balb/c mice	0–100 nM for 24–72 h 100 mg/kg/day	Reduction of cell growth in FLT3 mutant cells, Significant reduction in white blood cell count (WBC) and prolonged survival of FLT3-ITD transgenic mice	[63]
		MV4-11 (FLT3-ITD), RS4-11 (FLT3 wildtype) 4 primary patient blasts	20–100 nM for 24–48 h	Downregulation of FLT3, P-FLT3, P-AKT, P-ERK, P-STAT5 and induction of apoptosis in FLT3-ITD cells. Synergistic cytotoxic effects in cotreatment with 17-allylamino-demethoxygeldanamycin (17-AAG)	[64]
		65 primary patient blasts	0–10 μM for 72 h	Concentration-dependent cytotoxicity, inhibition of P-FLT3, synergistic effect with Ara-C	[67]
Lestaurtinib	FLT3	BaF3 cells with FLT3 (WT, -ITD, -D835Y), HEL, MV4-11 8 primary patient blasts	0–500 nM for 72 h	Reduction of cell growth in FLT3 expressing cells (wt and mutant), inhibition of P-FLT3, P-ERK, P-STAT5	[66]
CEP-701		BaF3/FLT-3-ITD Balb/c mice	10–20 mg/kg every 8 or 12 h	*In vivo* inhibition of FLT3 and prolonged survival in FLT3-ITD transgenic mice	
		65 primary patient blasts	0–10 μM for 72 h	Concentration-dependent cytotoxicity, inhibition of P-FLT3, synergistic effect with Ara-C	[67]
Tandutinib	FLT3, c-Kit, PDGFR	BaF3, Molm-13, Molm-14, HL-60, AML193, KG-1, THP-1	0.003–3 μM for 72 h	Inhibition of FLT3 phosphorylation, reduction of cell growth and induction of apoptosis in FLT-ITD cells	[71]

Table 1 (*Continued*)

Compound	Target	Cells/Xenograft models	Treatment schedule	Result	Refs.
MLN518 CT53518		BaF3/FLT3-ITD nude mice Balb/c FLT3-ITD murine BMT	20 or 60 mg/kg for 42d	Significant reduction in white blood cell count (WBC) and prolonged survival of FLT3-ITD transgenic or BMT mice	
KP372-1	FLT3, AKT, PDK1	KG-1, U937, TF-1 BaF3 with FLT3 (WT, -ITD, -D835G) 25 primary patient blasts	0–1 μM overnight	Reduction of cell growth, induction of apoptosis, inhibition of P-AKT, P-p70S6K, P-BAD, P-Foxo3a	[103]
Gefitinib	EGFR	HL-60, Kasumi, U937 8 primary patient blasts	0.078–10 μM for 6–96 h	Induction of morphologic, biochemical, and gene expression evidence of differentiation and inhibition of proliferation through an EGFR-independent mechanism	[123]
Others					
LY294002 and Wortmannin	PI3K	HL-60	30 μM, 100–500 nM	Induction of apoptosis, synergistic effect in combination with cytotoxic drugs, dephosphorylation of Akt/PKB	[102]
IC87114	PI3K (p110δ)	Bone marrow cells from newly diagnosed patients with AML	0.5–10 μM	Suppression of both constitutive and FLT-3-stimulated Akt activation in blasts Inhibition of AML cell proliferation	[105]
Rapamycin	mTOR	BaF3 with BCR/ABL or FLT3-ITD	0–10 nM	Reduction of cell growth, synergistic effect with other receptor tyrosine kinase (RTK) inhibitors such as PKC412 or Imatinib	[109]
		U937, HL-60, UT-7GM, UT-7EPO, HEL, KG-1 22 primary patient blasts	0–100 nM	Reduction of cell growth strongest in the most immature cell line (KG-1) Inhibition of constitutive p70S6K and 4E-BP phosphorylation and inhibition of cell growth in patient blasts	[106]
		U937	0–10 nM	Induction of apoptosis, reduction of Akt, Raf-1, MEK, ERK phosphorylation, synergistic effect in combination with UCN-01, a staurosporin inhibiting protein kinase C	[110]
RAD001	mTOR	U937 Primary patient blasts	0–100 nM	Minimal reduction in cell growth as a single agent but synergistic effect in combination with Ara-C	[49]
PD98059 and PD186352	MEK	OCI-AML3, HL-60, NB4 27 primary patient blasts	0–50 μM	Reduction of cell growth and ERK phosphorylation in cells with constitutive MAPK activation, cell cycle arrest and induction of apoptosis	[124]
		20 primary patient blasts	0–40 μM	Reduction of cell growth and ERK phosphorylation in cells with high ERK activity, cell cycle arrest and induction of apoptosis	[114]
		OCI-AML3, HL-60, KG-1	1.25 μM	Reduction of cell growth in cells with high ERK activity and sensitization to apoptosis induced by the small molecule Bcl-2 inhibitor HA14-1 or Bcl-2 antisense oligonucleotides	[113]
U0126	MEK	KG-1, HEL, M07e, TF-1, THP-1, 3 primary patient blasts	50 μM	Induction of apoptosis in some cell lines, synergistic effect with Ara-C observed in HEL cells Higher sensitivity to the inhibitor in 2/3 patient blasts compared to control cells	[116]
Tipifarnib	Farnesyl-transferase	12 primary patient blasts	25, 50 and 100 nM	Reduction of cell growth, synergistic effect in cotreatment with purine nucleoside analogs (PNA)	[119]
Zarnestra R115777					

ditions. However, overall survival rates are still unsatisfactory and are highly influenced by complications resulting from therapy. Improvement of the current therapeutic approaches is thus urgently needed and major advances are hoped to arise from a more targeted therapy strategy. The increasing understanding of the high impact of receptor tyrosine kinase signaling in AML and the discovery of specific alterations and deregulation of signaling cascades sparked interest in specifically targeting RTK signaling in this disease. Besides, the growing number of molecularly targeted drugs displaying promising results in other human cancers further brought the focus onto targeting RTK signaling in AML and led to a plethora of preclinical and clinical trials (Tables 1 and 2).

4.1. Receptor tyrosine kinases

Given the fact that a large number of AML cases show constitutive activation of one or more RTKs, the use of small molecule inhibitors targeting their tyrosine kinase activity appears to represent a great promise. The remarkable activity of imatinib mesylate (Gleevec) targeting the BCR-ABL fusion protein in chronic myeloid leukemia (CML) [50] further developed the interest in the field of molecular targeted therapies in leukemia. The major focus in AML has been directed towards FLT3 and c-Kit. The uncovering of other receptors important for AML cell biology and the development of a plethora of new small molecule inhibitors, however, has broadened the field of targeted research in this malignancy.

4.1.1. FLT3

FLT3 is a class III receptor tyrosine kinase expressed by immature hematopoietic cells. Its expression is important for the normal development of stem cells and the immune system [30]. The FLT3 ligand (FL) is a transmembrane protein that can be released as a soluble homodimeric protein. Both the membrane-bound, as well as the soluble form of FL are able to activate the tyrosine kinase activity of the receptor, and, synergistically with other hematopoietic growth factors, to induce proliferation of hematopoietic progenitor cells [51]. Expression of FLT3 is found in the majority of AML patients and a crucial role of this receptor in the survival and proliferation of leukemic blasts has been described in various studies [52]. Molecular alterations in the FLT3 receptor were first described in 1996 by Nakao et al. [28]. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis revealed longer transcripts within the juxtamembrane domain of FLT3 in a large proportion of AML patients. This finding led to the discovery of commonly occurring internal tandem duplications (ITDs) within that region. A plethora of subsequent studies uncovered an overall frequency of FLT-ITDs of about one-fourth of all cases of AML. More recently, in an additional cohort of AML patients, mutations within the activation loop of the kinase domain of FLT3 were found [29,53]. Point mutations commonly occur at position 835 (Asp835) and have been reported in around 7% of AML cases so far. Taken together, the global incidence rate of altered FLT3 function in AML patients caused either by FLT3-ITD, or by FLT3 activation loop mutation, represents almost one third of all cases. The high prevalence of activating mutations in this receptor therefore makes FLT3 one of the most interesting targets for molecularly based therapies (Fig. 1).

Indoline tyrosine kinase inhibitors are known to inhibit the tyrosine kinase activity of a number of RTKs, such as FLT3, c-Kit, PDGFR, VEGFR or the FGFR. Pre-clinical studies have examined the effect on the FLT3 receptor and its signaling and initial clinical trials have been aimed at evaluating their biological activity in AML patients [54–61]. SU5416 and the related compound SU5614 were shown to inhibit the kinase activity of both the wildtype FLT3, as well as the mutated receptor. Furthermore, both compounds inhibited downstream signaling via the MAPK and the STAT pathway, markedly decreased cellular proliferation and increased apoptosis in AML [54,55]. In phase II clinical studies evaluating SU5416 in elderly AML patients, or patients with relapsed and/or refractory disease, single agent treatment had only minimal clinical activity. Partial remission and hematopoietic improvement was seen, however, in a small cohort of patients and these improvements were of short duration [56,58,59]. A significant decrease in blast cell count down to undetectable levels and stable remission after administration of SU5416 has only been described in a single case study of a patient with relapsed AML [61]. SU11248 is another oral multitargeted kinase inhibitor that targets FLT3 together with other RTKs. Pre-clinical studies showed *in vitro* activity against the wildtype, as well as the mutated FLT3 receptor and *in vivo* activity against xenograft FLT3-ITD AML tumors in engraftment mouse models [62]. Phase I clinical trials underlined the action of SU11248 against the FLT3 kinase activity. Monotherapy of SU11248, however, only induced partial remission in a small number of AML patients for a short duration [57,60].

PKC412 is a small molecule FLT3 inhibitor that was identified and characterized by Weisberg et al. [63]. PKC412 selectively induces G1 cell cycle arrest and apoptosis in cell lines expressing mutant FLT3 by directly inhibiting its kinase activity. Progression of leukemia was inhibited in FLT3-ITD transduced mouse models making this inhibitor a valuable candidate for clinical trials in patients carrying mutant FLT3 receptors [63]. Subsequent pre-clinical studies highlighted the potential use of this inhibitor against FLT3 kinase activity and reported an effect of PKC412 on the phosphorylation of the downstream targets Akt, Erk and STAT5 [64]. A phase II clinical trial in a small cohort of patients showed significant clinical benefit and a 50% decrease in peripheral blast counts in most patients with mutated FLT3, in line with inhibition of receptor autophosphorylation [65].

Levis et al. characterized CEP-701, an indolocarbazole derivative with potent activity against autophosphorylation of wild-type and constitutively activated FLT3 [66]. CEP-701 inhibited the FLT3 downstream targets Erk and STAT5 and induced a cytotoxic effect in AML patient cells harboring

Table 2
Clinical trials in AML

Compound	Target	Trial type	Study arm and treatment regime	Outcome	Refs.
Tyrosine kinase inhibitors					
Imatinib STI-571	BCR-ABL, c-Kit, PDGFR	Phase II	21 patients; c-Kit$^+$ 600 mg/day up to 6 months	From minor response to complete remission	[81]
Gleevec		Phase II	18 patients 400 mg/day for a median of 60.5 days	No complete responses	[80]
		Case reports	5 patients; BCR-ABL, Ph$^+$ 300–600 mg/day	Complete cytogenic and molecular remission until end of the study	[82–86]
		Case report	3 patients; CBF, c-Kit mutant 800 mg/day for 30 days	No activity in patients with c-Kit kinase mutations, Abrogation of leukemic subclone carrying a c-Kit extracellular juxtamembrane mutation	[87]
		Case reports	2 patients; c-Kit$^+$; refractory or relapsed 600–800 mg/day	Complete remission	[88,89]
SU5416	FLT3, c-Kit, VEGFR	Phase II	33 patients, refractory or relapsed 145 mg/m^2 twice a week	Modest clinical activity as single agent; 3/33 partial remission, 1/33 hematologic improvement	[56,58]
		Phase II	43 patients, refractory or elderly; c-Kit$^+$ 145 mg/m^2 twice a week	Morphologic or partial response in 8 of the 25 evaluable patients (19%) but with short duration of remission	[59]
		Case report	1 patient; refractory, second relapse 145 mg/m^2 twice a week	Complete remission after 12 weeks of treatment	[61]
SU11248	FLT3, c-Kit, VEGFR, PDGFR	Phase I	15 patients 50 and 75 mg/day for 4 weeks	Morphologic or partial response in patients with FLT3 mutations (4/15) but with short duration of remission	[60]
		Phase I	29 patients 50–350 mg/day	Inhibition of FLT3 phosphorylation in 50% of FLT3-wildtype and 100% FLT3-mutant patients	[57]
PKC412	FLT3, c-Kit, VEGFR, PDGFR	Phase II	10 patients; FLT3 mutant 75 mg three times daily	Partial remission in one patient, significant clinical benefit in 14/20 patients (70%)	[65]
Lestaurtinib CEP-701	FLT3	Phase I/II	14 patients, refractory or relapsed 40 mg, 60 mg and 80 mg twice a day	Clinical response in 5/14 patients but with short duration of remission	[68]
		Phase II	29 elderly patients 60–80 mg twice daily for 8 weeks	Clinical response in 8/27 patients (30%), 3/5 FLT3 mutant and 5/22 FLT wildtype patients	[69]
PTK787 ZK222584	c-Kit, VEGFR, PDGFR	Phase I	63 patients 500–1000 mg/day	Broad range of repsonses in patients with combination with chemotherapy	[125]
Tandutinib	FLT3, c-Kit, PDGFR	Phase I	39 patients	No complete responses	[70]

Table 2 (*Continued*)

Compound	Target	Trial type	Study arm and treatment regime	Outcome	Refs.
MLN518			50–700 mg/day	Evidence of antileukemic effect in 2/8 patients at high doses	
CT53518					
Others					
RAD001	mTOR	Pilot study	9 patients, refract., relapsed, poor-risk 2 mg/day, 6 mg at day 1	Partial remission in 4/9 patients, stable disease in 1/9 patients	[106]
Sorafenib	c-Kit, Raf	Phase I	24 patients 200–800 mg/day	Effect on c-Kit pathway activation, antileukemic effect suggested	[117]
BAY43-9006					
Tipifarnib	Farnesyl-transferase	Phase I	25 patients, poor-risk 100–1200 mg/day	Complete remission in 2/25 patients, partial remission in 7/25 patients	[120]
Zarnestra R115777		Phase II	158 patients, poor-risk 600 mg twice daily	Complete remission in 22/138 patients, partial remission or hematologic improvement in 15/138 patients	[121]
BMS-214662	Farnesyl-transferase	Phase I	19 patients, refractory or relapsed 42-300 mg/m^2 once weekly	Complete remission (but incomplete platelet recovery) in 2/19 patients, partial remission in 2/19 patients	[122]

FLT3-ITDs. Furthermore, CEP-701 prolonged survival in a mouse model of FLT3-ITD leukemia [66]. A recent study substantiated the potential of CEP-701 in reducing cell viability and FLT3 phosphorylation in a large number of patient cells [67]. *In vivo* hematological activity of CEP-701 has been studied in clinical phase I/II trials. Treatment of patients with refractory, relapsed, or poor prognosis AML expressing FLT3-activating mutations with CEP-701 revealed biological activity and a measurable clinical response including reduction in peripheral and bone marrow blood blasts in some patients [68]. The clinical response was more pronounced in patients with mutated FLT3 than in those expressing the wild-type receptor [69].

Recently, tandutinib (MLN518) was identified as a novel, relatively specific FLT3 antagonist [70]. Pre-clinical studies showed activity against FLT-ITD autophosphorylation and inhibition of AML cell proliferation *in vitro* [71]. A clinical phase II study reported evidence of antileukemic activity with a decrease in both peripheral and bone marrow blast count in a small cohort of patients treated with tandutinib [70].

4.1.2. c-Kit

c-Kit encodes another transmembrane protein with tyrosine kinase activity and is a proto-oncogene thought to play an important role in normal hematopoiesis. c-Kit expression has been detected in a large number of hematopoietic cell lines, in primary blasts of human AML patients, but to a much lower level in normal bone marrow cells [72]. Tyrosine phosphorylation and activation of c-Kit is induced by binding of stem cell factor (SCF) and results in proliferation of human leukemia blast cells in a substantial fraction of AML cases. The constitutive, ligand-independent activation of c-Kit found in leukemic cells suggests that this receptor is involved in the excessive proliferation and aberrant differentiation of these cancer cells [73]. Moreover, a direct correlation has been found between the levels of c-Kit tyrosine phosphorylation and the proliferation rate in AML cell lines [36]. Mutations in c-Kit are thought to be exclusively associated with core binding factor leukemias (CBF-AML). In addition to mutations within the tyrosine kinase domain of c-Kit at position 816, insertions and deletions have been described [74,75].

Imatinib, also known as STI571 or Gleevec, has been shown to be a very promising small molecule inhibitor targeting the tyrosine kinase activity of the BCR-ABL fusion protein commonly found in chronic myeloid leukemia. Moreover, the inhibitor was shown to effectively inhibit the platelet-derived growth factor receptor (PDGFR) as well as c-Kit [76]. Imatinib prevented autophosphorylation of c-Kit and activation of the downstream signal transducers MAPK and Akt. Furthermore, treatment of an AML cell line expressing the BCR-ABL fusion protein induced apoptosis and significantly increased the sensitivity to chemotherapeutical agents [77]. In c-Kit-positive primary AML patient blasts, however, Imatinib exerted only marginal effects on cell growth [78]. Nevertheless, a pre-clinical study analyzing

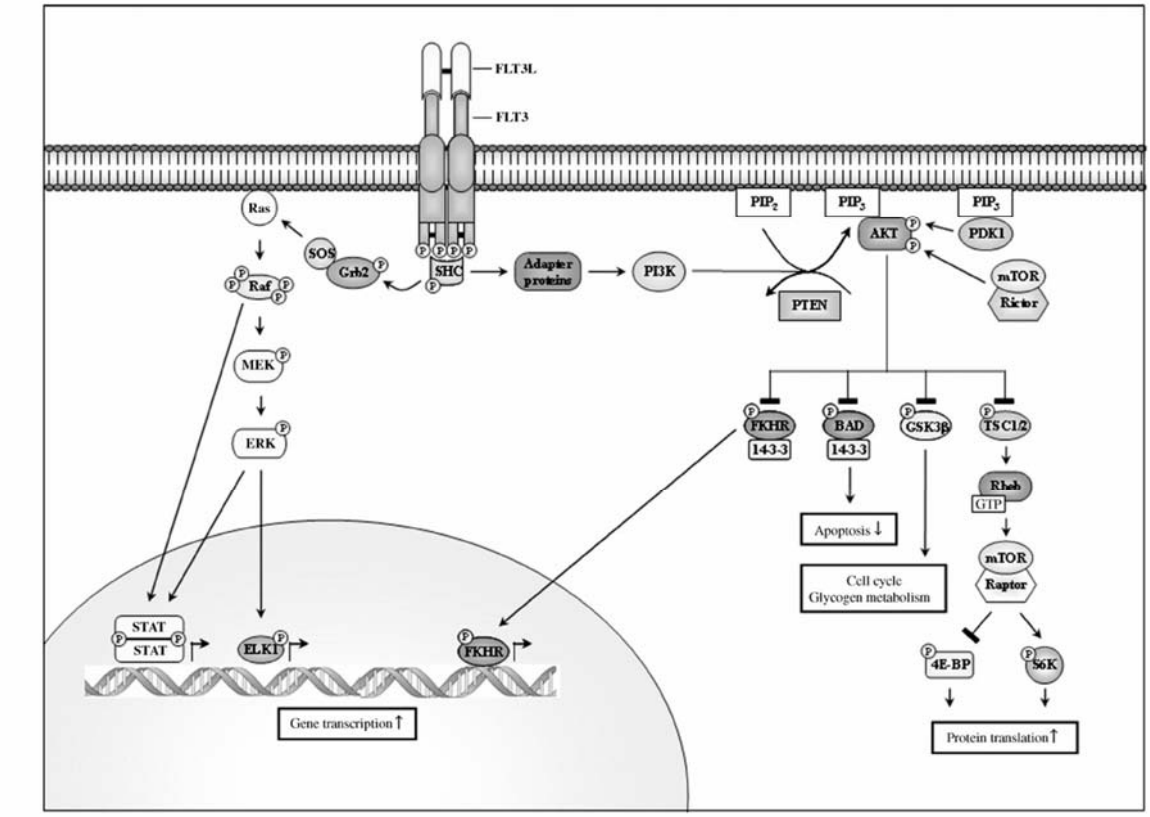

Fig. 1. Overview of signaling from the activated FLT3 receptor. Upon binding of the FLT3 ligand (FLT3L), the transmembrane receptor FLT3 is activated by autophosphorylation, creating binding sites for the Src-homology collagen protein (Shc). Signals are transduced via the mitogen-activated protein kinase (MAPK) or the phosphoinositide 3-kinase (PI3K) pathway. Key upstream regulators of MAPK signaling include growth factor receptor-bound protein 2 (Grb2), Son of Sevenless (SOS), Ras, Raf, MAPK/Erk kinase (MEK), extracellular signal-regulated kinase (ERK). Downstream targets include signal transducers and activators of transcription (STAT) and ELK1. Activation of the PI3K pathway is initiated by recruitment of the PI3K complex to the phosphorylated receptor through interactions with a number of proteins, including CBL, SH2-domain-containing inositol phosphatase (SHIP) or SH2-domain-containing protein tyrosine phosphatase 2 (SHP2). Downstream signals are transduced via phosphoinositide-dependent kinase 1 (PDK1), the mammalian target of rapamycin (mTOR)-Rictor complex, protein kinase B/Akt (AKT), forkhead (FKHR), BAD, glycogen synthase kinase-3β (GSK3β), tuberous sclerosis complex (TSC1/2), Ras homologue enriched in the brain (Rheb), the mTOR-Raptor complex, 4E-binding protein (4E-BP) and ribosomal protein S6 kinase (S6K).

cells expressing c-Kit mutants cloned from AML patients reported a dose-dependent increase in apoptosis upon treatment with the inhibitor [79]. In a first clinical phase II trial of patients with refractory or recurrent AML, Imatinib as single agent did not induce any significant beneficial responses [80]. A later phase II pilot study including a small cohort of c-Kit-positive patients showed high efficacy of Imatinib resulting in the inhibition of c-Kit tyrosine phosphorylation and promising results in a subset of patients meeting the criteria for complete hematological remission [81]. Consistently, different case studies have described a potential benefit of Imatinib treatment for c-Kit-positive AML patients and carriers of specific c-Kit mutations [82–89].

4.1.3. IGF-IR

The insulin-like growth factor receptor I (IGF-IR) is a transmembrane tyrosine kinase widely expressed in many human tissues and cell types, with high homology to the insulin receptor (IR). Activation of the receptor is achieved by binding of the insulin-like growth factors (IGFs) to the extracellular domain, triggering autophosphorylation of three tyrosine residues within the kinase domain [90]. In AML, signaling by the IGF-IR has not yet been extensively studied, however, expression of the IGF-IR was reported in human AML cells [91,92]. While mutations resulting in constitutive tyrosine kinase activity of the IGF-IR have not been described to date, over-expression of the receptor and/or the establishment of autocrine loops involving the ligands IGF-I and IGF-II have been reported in various human cancers [93]. The importance of IGF-I as a growth factor in combination with other cytokines has been described in human AML cells [46]. A recent study further underlined the importance of IGF-IR signaling by showing an association between increased expression of IGF-I and resistance to the chemotherapeutical

agent Cytarabine (Ara-C) in leukemia [94]. Gene expression profiling of Ara-C-resistant human myeloid leukemia cells revealed an up-regulation of IGF-I. Subsequent analysis of AML cases revealed higher IGF-I expression in patients with refractory disease after Ara-C therapy compared to patient cells analyzed at diagnosis [94]. Consistently, a role of autocrine IGF-I production in drug resistance was previously suggested in an AML cell line [47]. In addition, IGF-I signaling is known to play a crucial function in other hematological malignancies such as multiple myeloma [95], and several anti-IGF-IR experimental therapies have been shown to inhibit multiple myeloma proliferation *in vitro* and *in vivo* [96]. Blocking the IGF-IR in the Ara-C resistant myeloid cell line described above inhibited cell growth and led to the induction of apoptosis, suggesting that the IGF-IR and its downstream signaling pathways may provide valuable novel targets to overcome Ara-C resistance in AML [94].

The increasing understanding of the importance of the IGF-IR signaling pathway in AML cell proliferation, viability and drug-resistance makes this system an interesting new molecular target for cancer therapy. Promising small molecule inhibitors have been generated and a wide variety of strategies are now available to target IGF signaling [97].

4.2. Downstream signal transducers

Targeting pathways downstream of RTKs is another approach to down-regulate an overactivated signaling system with significant therapeutic advantages. The growing understanding of the complexity of RTK signaling networks has indeed revealed a number of promising target proteins contributing to altered signaling in cancer cells. As described above, deregulation of RTKs frequently induces over-activation of the PI3K/Akt, Ras/MAPK and JAK/STAT cascades in AML. Inhibitors targeting various signal transducer proteins including Akt and mTOR, Ras and MEK have been developed and are being tested in preclinical studies in various human cancers including AML.

4.2.1. PI3K/Akt

The phosphoinositide 3-kinase (PI3K) pathway is essential for different physiological processes including transcription, translation, cell cycle progression and apoptosis. The PI3K cascade is the signaling system most frequently targeted by genetic alterations in human cancer, along with p53 and the retinoblastoma pathway. Genomic aberrations such as amplifications, rearrangements and mutations induce activation of the pathway, thus providing the tumor cells with a strong growth advantage [98]. The PI3Ks are a family of eight enzymes in humans, which are subdivided into 3 classes (I–III), based on sequence homology and substrate specificity [23]. Class I_A and class II PI3Ks transduce signals from activated RTKs. These PI3Ks are also activated by oncogenic mutants of Ras, such as N- and K-Ras, which are found in AML. Several downstream targets of PI3K transduce their proliferative and anti-apoptotic signals, including Akt/PKB, phosphoinositide-dependent kinase-1 (PDK-1), forkhead transcription factors (FKHR), glycogen synthase kinase-3β (GSK-3β), tuberous sclerosis complexes 1 and 2 (TSC1, TSC2), BAD, Ras homologue enriched in the brain (Rheb), mammalian target of rapamycin (mTOR), S6 kinase (S6K), and 4E-binding protein (4E-BP). The importance of the PI3K/Akt axis in AML cell progression has been highlighted by several studies [49,99–101]. Constitutive activation of PI3K was found in more than 50% of AML cases and the activation of Akt was significantly higher in spontaneously proliferating AML cells than in cells that did not proliferate spontaneously [100]. Moreover, constitutive phosphorylation of Akt at Ser473 negatively correlated with the overall survival rate of AML patients [99].

The inhibition of PI3Ks by the generic inhibitor LY294002 resulted in reduced Akt kinase activity, dephosphorylation of Akt and BAD and an increase in apoptosis in AML cell lines [49,101]. PI3K inhibition in primary AML blasts resulted in significantly reduced clonogenic growth [101]. Moreover, inhibition of the pathway sensitized AML cells to chemotherapy-induced apoptosis [102]. A recent study examined the antileukemic effect of the novel small-molecule multiple kinase inhibitor KP372-1 in AML [103]. KP372-1 potently inhibited the kinase activity of Akt, PDK1 and FLT3 in a dose-dependent manner. The inhibitor induced pronounced apoptosis in AML cell lines and primary blasts. Furthermore, KP372-1 decreased the colony-forming ability of primary AML blasts with minimal cytotoxic effects on normal progenitor cells [103]. The finding that normal hematopoietic progenitor cells were less affected by inhibitors targeting PI3K signaling than AML cells was previously described by others [49,101] and further supports the feasibility of targeting this fundamental signal transduction network for AML therapy. The use of generic non-selective inhibitors such as LY294002 or wortmannin, however, has been shown to cause severe side effects in xenograft models [104] and the identification of more selective inhibitors is required. Recently, IC87114, a class I_A PI3K p110δ-selective inhibitor, was shown to efficiently suppress constitutive as well as FLT3-stimulated activation of Akt in AML blasts to the same extent as the non-selective generic PI3K inhibitor LY294002 [105]. Moreover, the isoform-selective inhibitor suppressed AML cell proliferation without affecting the proliferation of normal progenitor cells.

4.2.2. mTOR

The mammalian target of rapamycin (mTOR) is a key regulator of growth and survival and is activated by various RTK receptors. Once activated, the serine/threonine kinase phosphorylates its downstream targets, the ribosomal S6K and 4E-BP. Aberrant activation of the mTOR signaling pathway in AML cells is supported by the finding of constitutive phosphorylation of S6K and 4E-BP in the majority of AML samples [106].

Rapamycin is a well known inhibitor of mTOR and is used clinically as an immunosuppressant and anti-proliferative

agent [107,108]. Studies evaluating the effect of rapamycin in AML cells have reported marked activity of the inhibitor in down-regulating phosphorylation of S6K and 4E-BP [106,109,110] and inhibiting cell growth by blocking the cell cycle, particularly in very immature AML cell lines [106]. Treatment of primary AML blasts with the mTOR inhibitor impaired their clonogenic properties, while normal hematopoietic progenitors were not affected. Moreover, rapamycin synergistically induced apoptosis in conjunction with protein kinase inhibitors [109,110]. A pilot clinical study comprising a small number of patients with refractory, relapsed or poor prognosis AML showed significant responses suggesting that rapamycin could be of clinical interest for AML treatment. Partial remission or stabilization of the disease was reported in five out of nine patients analyzed [106]. Evaluation of RAD001 (Everolimus), a rapamycin derivative, revealed only marginal effects on AML cell growth as a single agent. Combined treatment together with the chemotherapeutical agent Ara-C, however, significantly enhanced the response of AML cells to the cytotoxic drug [49].

4.2.3. RAS/RAF/MEK/ERK

The mitogen-activated protein kinase (MAPK) signaling cascade is yet another important system that integrates extracellular stimuli and transduces them to cellular responses, such as proliferation, differentiation and survival. RTKs signal through activation of the small GTP-binding protein Ras via the adapter molecule Grb2 and the guanine nucleotide exchange factor Son of Sevenless (SOS). Sequential stimulation of the cytoplasmatic proteins Raf, MEK and Erk, collectively known as MAPKs, finally results in the regulation of gene transcription by ELK1 and the STAT proteins [26]. The MAPK pathway was shown to be constitutively activated in a large number of AML cells, suggesting a pivotal role for this cascade in leukemogenesis [111,112]. N-Ras, but also K-Ras or H-Ras, are frequently mutated in AML cells causing dysregulation and activation of the signaling system [37,38]. As described above, altered signaling emanating from FLT3 receptors containing internal tandem duplications also relies, at least in part, on activation of MAPKs, thereby inducing autonomous growth of myeloid cell lines and primary AML blasts [39]. Moreover, the BCR-ABL chimeric oncoprotein that results from chromosomal translocation is yet another trigger for alterations in the MAPK pathway [40].

PD98059 and PD184352 are small molecule inhibitors that strongly reduce MAPK activity and profoundly impair growth and survival of AML cells [113]. The primary effect of the inhibitors included cell cycle arrest followed by apoptosis in a significant percentage of leukemic blasts [114]. The inhibitors abrogated the clonogenic properties of primary AML cells, but had only minimal effects on normal hematopoietic progenitors. Moreover, impairment of MAPK signaling sensitized leukemic cells to spontaneous, as well as drug-induced apoptosis [113,115]. U0126, another potent MEK inhibitor, led to a highly significant induction of apoptosis in some AML cell lines and blasts, while no apparent responses were seen in others [116]. Interestingly, a particularly pronounced effect on the most primitive types of leukemia, which are often found to be the most resistant to standard chemotherapy, was observed. A possible synergistic effect in combination with the chemotherapeutical agent Ara-C was also observed. Moreover, control cells were completely insensitive to the inhibitor [116]. A phase I clinical trial addressed the question of efficacy of Sorafenib (BAY43-9006), a Raf-1 kinase inhibitor, in AML patients [117]. Although no significant inhibition of the MAPK cascade, as assessed by phosphorylation of Erk, could be detected, c-Kit-mediated pathway activation was largely abolished upon treatment with the inhibitor. In fact, further analysis revealed an almost complete inhibition of c-Kit activity at high doses of the inhibitor, putting c-Kit forward as another target of Sorafenib [117].

Another approach to target the MAPK pathway is based on interfering with Ras protein function. Proper membrane localization of Ras proteins is a critical step for successful signal transduction. Binding of Ras to the plasma membrane, which is required for its full biological activity, is accomplished by diverse post-translational modifications of the protein, which are catalyzed by specific enzymes. Various inhibitors of these enzymes have been generated in order to impair the post-translational modification steps. The most significant progress has been made in the identification and characterization of farnesyltransferase inhibitors (FTIs). Treatment of tumor cells with these inhibitors results in various effects including alteration of cell cycle progression, induction of apoptosis, changes in cell morphology and inhibition of anchorage-independent growth [118]. Several FTIs have entered clinical trials for AML with promising results. Zarnestra (R115777) significantly inhibited the colony growth of human AML blasts and induced apoptosis. In combination with other drugs the effect of the FTI was even more pronounced [119]. Phase I and II clinical trials have described a promising biological activity of Zarnestra, inducing antileukemic responses in a subgroup of patients treated with the inhibitor [120,121]. Accordingly, evaluation of the FTI BMS-214662 showed strong evidence of antileukemic activity and a good tolerability of the compound in a cohort of patients with relapsed or refractory disease [122].

5. Conclusions and perspective

A better understanding of the mechanisms underlying tumor formation has resulted in the development of both more specific inhibitors, as well as combinatorial therapies, in which multiple signaling pathways are being targeted. The new era of cancer treatment strategies using highly specific small-molecule inhibitors has not only proven that these inhibitors can be as effective as traditional therapies, but also that the incidence of adverse side effects can be greatly reduced. Extensive research aimed at identifying promising

new molecular targets has placed RTKs in the front ranks. The wide distribution of these proteins in different human cancers, along with the fact that they are frequently mutated or activated in tumors makes them attractive targets. At the same time, the ubiquitous expression of certain RTKs and their crucial role in normal development and cell survival implies certain risks associated with the use of tyrosine kinase inhibitors. In order to prevent unpredictable responses to newly developed drugs, a detailed understanding of the structure and biological role of these proteins is essential. Great progress has been made in understanding the pathogenesis of AML, revealing a number of promising targets, such as FLT3 and c-Kit. Despite the fact that clinical trials have corroborated the feasibility of using these newly developed targeted agents in AML and have demonstrated acceptable tolerability in patients, none of these inhibitors has entered into the routine treatment for leukemia yet. Moreover, due to the heterogeneity of the disease, the inhibitors currently in clinical trials have only shown satisfactory results in certain subgroups of patients. Therefore, a number of other molecular targets remains yet to be discovered. As more and more targets are identified, a broader selection of specific agents will be available that could be combined to achieve optimal inhibition of AML progression. Together with a detailed knowledge of the molecular fingerprint of individual patient tumors, individualized treatment strategies could be developed, which would hopefully lead to a better outcome for a large group of AML patients. The IGF-IR provides an example for an RTK that is just starting to emerge as a novel target in AML. As the IGF-IR is already known to be a validated candidate in other human cancers, the role of this receptor in AML is the focus of current research.

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7 Curriculum Vitae

CURRICULUM VITAE

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Marital status: unmarried
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EDUCATION

DISSERTATION (DR. SC. NAT)	2004-2008	UNIVERSITY CHILDREN'S HOSPITAL ZURICH

Investigation of the role phosphatidylinositol-3 kinases play in the development of neuroblastoma and glioblastoma.
Supervisor: Dr. Alexandre Arcaro

DIPLOMA IN BIOLOGY (DIPL. NATW. ETH)	1999-2004	SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH (ETH ZURICH)

FINAL EXAMS: Cell Biology, Biochemistry, Genetics, Ecotoxicology and Immunology

DIPLOMA THESIS: Investigation of the role of cell cycle inhibitors, mainly p21, in regulating Schwann cell proliferation.
Supervisors: Dr. Suzana Atanasoski and Prof. Ueli Suter (Institute for Cell Biology, ETH Zurich).

SWISS MATURA	1992-1999	GYMNASIUM HOHE PROMENADE ZURICH

Matura Type D with a main focus on languages (French, English, Spanish).

PROFESSIONAL EXPERIENCE

DISTRIBUTION CONTRACT ADMINISTRATOR	04/2004-09/2004	BVS INTERNATIONAL N.V,
WAITRESS	02/2002-09/2004	LAKESIDE CASINO ZÜRICHHORN
TRANSLATION ASSISTANT	12/2001-01/2002	DOTPROMOTION AG
ADMINISTRATIVE ASSISTANT	02/1999-07/2002	PUEBLO FILM AG, ZURICH
TEMPORARY ASSISTANT	02/1999-05/1999	SWISSAIR

LANGUAGES

ENGLISH AND GERMAN:	Mother tongues
FRENCH:	Good knowledge
SPANISH:	Basic knowledge

PROFESSIONAL ASSOCIATIONS

USGEB	Union Schweizerischer Gesellschaften für Experimentelle Biologie
SSB	Schweizerische Gesellschaft für Biochemie

POSTERS AND PRESENTATIONS

POSTERS

2007

British Association for Cancer Research
Cell Signalling and Novel Cancer Therapeutics, London
Targeting the phosphoinositide 3-kinase isoform p110δ impairs growth and survival in neuroblastoma cells
Boller D, Schramm A, Doepfner KT, Shalaby T, Eggert A, Grotzer MA, Arcaro A

USGEB –SSN-SSBP Meeting 2005, Kongresszentrum Basel
PI3-kinase signaling in glioblastoma
D. Boller, K. Frei, A. Arcaro

6th Day of Clinical Research, University Hospital Zurich
Phosphatidylinositol 3-kinase Signaling in Glioblastoma
D. Boller, K. Frei, A. Arcaro

2006

Targeting the Kinome, Congress Center Basel
Signaling by Specific Phosphatidylinositol-3 kinase Isoforms in Human Neuroblastoma
D. Boller, T. Shalaby, M.A. Grotzer, A. Arcaro

4th Swiss Apoptosis Meeting, Inselspital Berne
Signaling by Specific Phosphatidylinositol-3 kinase Isoforms in Human Neuroblastoma
D. Boller, T. Shalaby, M.A. Grotzer, A. Arcaro

5th Day of Clinical Research, University Hospital Zurich
Signaling by Specific Phosphatidylinositol-3 kinase Isoforms in Human Neuroblastoma
D. Boller, T. Shalaby, M.A. Grotzer, A. Arcaro

Poster Presentation, University Children's Hospital Zurich
Signaling by Specific Phosphatidylinositol-3 kinase Isoforms in Human Neuroblastoma
D. Boller, T. Shalaby, M.A. Grotzer, A. Arcaro

2005

4th Day of Clinical Research, University Hospital Zurich
Signaling by Specific Phosphatidylinositol-3 kinase Isoforms in Human Glioblastoma and Neuroblastoma Cell Proliferation, Chemoresistance and Metastasis
D. Boller, A. Arcaro

Poster Presentation, University Children's Hospital Zurich
Signaling by Specific Phosphatidylinositol-3 kinase Isoforms in Human Glioblastoma and Neuroblastoma Cell Proliferation, Chemoresistance and Metastasis
D. Boller, A. Arcaro

USGEB –SSN-SSBP Meeting 2005, ETH Zürich
PI3- kinases as molecular targets for pediatric neuroblastoma and acute myeloid leukemia
D. Boller, K. Doepfner, A. Arcaro

USGEB –SSN-SSBP Meeting 2005, ETH Zürich
Targeting IGF-IR in human neuroblastoma: potent antitumor activity of the novel inhibitor NVP-AEW541
A.S. Guerreiro, **D. Boller**, T. Shalaby, M.A. Grotzer, A. Arcaro

ORAL PRESENTATIONS

2006	SPOG-Forschungsratssitzung *Signaling by Specific PI3- kinase Isoforms in Neuroblastoma Cell Proliferation, Chemoresistance and Metastasis* **D. Boller,** T. Shalaby, M.A. Grotzer, A. Arcaro
2005	Journée des Jeunes Chercheurs en Pédiatrie, Inselspital Bern *Signaling by Specific PI3- kinase Isoforms in Neuroblastoma Cell Proliferation, Chemoresistance and Metastasis* **D. Boller,** A. Arcaro

PUBLICATIONS

RESEARCH ARTICLES

1. **Boller D**, Schramm A, Doepfner KT, Shalaby T, Eggert A, Grotzer MA, Arcaro A (2007): Targeting the phosphoinositide 3-kinase isoform p110δ impairs growth and survival in neuroblastoma cells; *Clinical Cancer Research (in press)*

2. Arcaro A, Doepfner KT, **Boller D**, Guerreiro AS, Shalaby T, Jackson SP, Schoenwaelder SM, Delattre O, Grotzer MA, Fischer B (2007): Novel role for insulin as an autocrine growth factor for malignant brain tumour cells; *Biochem J. 2007 Aug 15;406(1):57-66*

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1. Doepfner KT, **Boller D**, De Laurentiis A, Guerreiro AS, Marinov M, Arcaro A (2007): Recent patents of gene sequences relative to the phosphatidylinositol 3-kinase/Akt pathway and their relevance to drug discovery; *Recent Patents in DNA and Gene Sequences 2007, 1, 9-23*

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3. Guerreiro AS, **Boller D**, Doepfner KT, Arcaro A (2006): IGF-IR: Potential role in antitumor agents; *Drug News Perspect. 2006 Jun;19(5):261-72*

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